Antimicrobial resistance and molecular characterization of *Escherichia coli* isolated from Ontario chickens

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Abstract

In Canada, there is a lack of data regarding broad-spectrum β-lactamase producing *E. coli* in chickens. This thesis aimed to identify and characterize broad-spectrum β-lactamase producing *E. coli* in chickens coming from small and large-scale flocks.

Chickens coming from small-scale flocks are not included as part of the Canadian national antimicrobial resistance surveillance program. We performed our study on this understudied bird population to identify broad spectrum β-lactamase producing *E. coli*. Following PCR and DNA sequencing in order to identify resistance genes, isolates were further characterized by PFGE to determine their relatedness. A subset of samples was subjected to MLST to determine if human resistance pandemic clones (*E. coli* ST131) were present in this chicken population. CTX-M-1 and CMY-2 were identified as predominant ESBL and AmpC β-lactamase genes. The population was genetically heterogeneous, only 32 out of 226 isolates formed clusters. None of the isolates were tested by MLST were ST131.

We also conducted a comparative study of archived samples from chickens raised in large-scale flocks; stored samples were screened for 3rd generation cephalosporin resistance using CHROMagar ESBL (selective media). The results of this testing were compared to the findings of CIPARS to determine the value of including selective media in resistance surveillance programs. Following antimicrobial susceptibility testing by microbroth dilution, isolates resistant to 3GC were screened for ESBL and AmpC producing genes using PCR. The majority of the isolates were multi-drug resistance. Like chickens from small-scale flocks, CTX-M-1 and CMY-2 were found to be the predominant broad-spectrum β-lactamases.

This is the first systematic documentation of the types of β-lactamases produced by *E. coli* isolated from Canadian chickens. We found chicken from both small and large flocks carried broad-spectrum β-lactamase genes conferring resistance to extended-spectrum cephalosporins. Further, our findings suggest that resistance is spreading in chickens via mobile genetic elements rather than clonal transfer. Because different β-lactamases were identified among poultry isolates than are common among human clinical isolates, our findings suggest that *E. coli* from Canadian chicken may pose a lower risk to public health than previously thought. More research is required to evaluate the magnitude of the risk of poultry borne drug resistant *E. coli* to public health.

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List of Abbreviations

3GC	3rd Generation Cephalosporin
AMR	Antimicrobial resistance
AMU	Antimicrobial use
CDC	US center for Disease Control and Prevention
CFC	Chicken Farmers of Canada
CFO	Chicken Farmers of Ontario
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CR	Conserved region
CRE	Carbapenem-resistant Enterobacteriaceae
CRO	Carbapenem-resistant organisms
DNA	Deoxy-ribo nucleic acid
ESBL	Extended-spectrum β-lactamase
ExEPC	Extra-intestinal pathogenic *E. coli*
GAS	Group A *Streptococcus*
IMP	Imipenemase
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo β-lactamase
MHA	Muller-Hinton agar
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo β-lactamase
OMAFRA	Ontario Ministry of Agriculture, Food Safety and Rural Affairs
OXA	Oxacillinase
pAmpC	Plasmid-mediated AmpC

PBPs	Penicillin-binding proteins
PFGE	Pulsed-field gel electrophoresis
PHAC	Public Health Agency of Canada
UTI	Urinary tract infection
VIM	Verona integron encoded metallo β-lactamase
VRE	Vancomycin-resistant Enterococci

1 Introduction and Literature review

1.1 Antibiotic resistance

1.1.1 General introduction to the problem of antimicrobial resistance

The discovery of antibiotics was a revolutionary achievement for both human and veterinary medicine. Since the discovery, antimicrobials have cured humans and animals from bacterial infections (Byarugaba, 2010). Antimicrobials can be defined as any natural, synthetic or semisynthetic origin substance which kill or inhibit the growth of microorganisms (Giguère, 2013; Guardabassi and Courvalin, 2006; Maartens et al., 2011). In contrast, antibiotic refers to a low molecular weight substance produced by microorganisms which act against another microorganism at low concentrations (Giguère, 2013; Guardabassi and Courvalin, 2006; Maartens et al., 2011). The term antibiotic has been used interchangeably with the term antimicrobial in many instances (Giguère, 2013).

The first antibiotic was penicillin discovered by Alexander Fleming in 1928 and was first used therapeutically in the 1940s. However, treatment failures and bacteria resistant to penicillin were first noticed immediately after the discovery of penicillin (as reviewed in Aminov, 2010; Byarugaba, 2010; Ventola, 2015). Even Alexander Fleming warned that bacteria could become resistant to these remarkable drugs in his Nobel Prize speech in 1945 (WHO, 2014). Indeed, each new antibacterial drug development has been followed by the detection of resistance to it. Antibiotic resistance is defined as the ability of microbes to resist the effects of drugs, as a result the drugs become ineffective to neither kill nor inhibit the microbes (CDC, 2015; WHO, 2014). Antimicrobial resistance in bacteria results from selection pressures applied by antimicrobial use

(McDermott et al., 2002; WHO, 2014). Use of antibiotic can trigger the antimicrobial resistance by exerting selection pressure on bacterial strains (McDermott et al., 2002).

The emergence and rapid spread of antimicrobial resistance is now a global concern (Laxminarayan et al., 2013; Ventola, 2015; WHO, 2014). In addition to the prospect of untreatable infections, antimicrobial resistance results in higher economic costs due to longer hospital stays in infected patients, the requirement for additional diagnostics and more expensive drugs. In the United States, the economic burden from excess medical expenditures due to antimicrobial resistance is estimated to be approximately $20 billion each year (Marston et al., 2016). In the United States, more than 2 million infections and approximately 23,000 deaths are associated with antimicrobial resistant organisms annually (CDC, 2015), while approximately 25,000 annual deaths are attributed to resistance in Europe (Marston et al., 2016).

1.1.2 Mechanism of resistance and mode of transfer

Antibiotics work on bacterial cells in different ways and understanding of these mechanisms along with the chemical nature of antimicrobial agents is important to have a better understanding of antimicrobial resistant developments (**Figure 1.1**). Five different mechanisms of action are briefly described below:

Inhibition of the cell wall synthesis: β-lactam drugs (eg: penicillin, cephalosporin etc.) and the glycopeptides (eg: vancomycin) act in this way (Mcdermott et al., 2003; Wright, 2010). The β-lactam drugs possess the β-lactam ring which is the functional unit of this group of antibiotics. The β-lactam ring binds with bacterial penicillin-binding proteins (PBPs) located in bacterial cell membrane thus prevent cross-linking in peptidoglycan synthesis and inhibit the

bacterial cell wall synthesis (Džidić et al., 2008; Livermore and Woodford, 2006; Mcdermott et al., 2003; Tenover, 2006).

Inhibition of protein synthesis: In bacteria, ribosomes play role in protein synthesis and they are structurally different than eukaryotic ribosomes (Walsh, 2000). Some antibiotics (eg: macrolides, aminoglycosides, tetracyclines, chloramphenicol) can interfere with bacterial ribosome function by binding with its structural subunits (either 30s or 50s) and thus inhibiting protein synthesis. Tetracyclines, aminoglycosides bind to the 30s ribosomal subunit while chloramphenicol, macrolides bind to 50s subunit (Džidić et al., 2008; Tenover, 2006).

Inhibition of nucleic acid synthesis: Quinolones and fluoroquinolones inhibit nucleic acid synthesis by binding to DNA gyrase and DNA topoisomerase IV. These enzymes are responsible for relaxing and supercoiling the DNA within a cell. Binding with these enzymes can interfere with the DNA replication process. Another drug rifampicin binds bacterial DNA-dependent RNA polymerase and thus inhibits RNA synthesis (Džidić et al., 2008; Tenover, 2006; Walsh, 2000).

Inhibition of folate synthesis: Sulfonamides and trimethoprim act by interfering with folate synthesis. Sulfonamides inhibit the bacterial folate synthesis by excluding para-aminobenzoic acid (PABA) incorporation into the folic acid molecule through competing for the enzyme dihydropteroate synthetase while trimethoprim directly inhibits the dihydrofolate reductase enzyme (Džidić et al., 2008; Mcdermott et al., 2003; Tenover, 2006). Most of the time sulfonamides and trimethoprim are used combined for synergism (Smith and Powell, 2000).

Inhibition of cell membrane function: Polymyxin and daptomycin kill the bacteria in this way. This is not a well-defined mechanism; it is hypothesized that polymyxins increase the

bacterial permeability resulting in leakage of bacterial content which leads to bacterial cell death. Daptomycin binds to the bacterial cell membrane and can kill the bacteria because of potassium efflux from the bacterial cell (Džidić et al., 2008; Tenover, 2006).

Resistance can be either intrinsic or acquired. Intrinsic resistance defines the lack of bacterial inherent structural or functional properties which is required for the antimicrobial to act (Blair et al., 2015; Guardabassi and Courvalin, 2006; Mcdermott et al., 2003). Intrinsic resistance is constitutive to bacterial genera or species without the requirement for the acquisition of novel genetic material. An example of intrinsic resistance is the lipopeptide daptomycin which is effective against Gram-positive bacteria but is not effective against Gram-negative bacteria due to differences in cell membrane structure (Blair et al., 2015). Bacteria can acquire antimicrobial resistance either by chromosomal mutation and acquisition of resistance genes by horizontal transfer (Blair et al., 2015; Catry et al., 2003; Džidić et al., 2008; Guardabassi and Courvalin, 2006; Ruppé et al., 2015). Mutation in chromosomal genes can occur in a variety of ways such as spontaneous mutation, hypermutator and adaptive mutagenesis (Džidić et al., 2008). Horizontal gene transfer is the transfer of genetic material between individual bacteria of same species or different species. This mechanism of gene transfer is one of the important means of dissemination of antibiotic resistance genes among bacteria. Different transposable genetic elements such as plasmids, transposons, gene cassettes play an important role in carrying resistance genes and three major mechanisms by which bacteria transfer gene horizontally are conjugation, transformation and transduction (Babic et al., 2006; Drawz and Bonomo, 2010; Huddleston, 2014; Mcdermott et al., 2003; Wright, 2005).

Conjugation: During conjugation, two bacterial cells (donor and recipient) come into contact with each other and they form a channel via pilus or pore that allows the transfer of

genetic material from donor cell to recipient. Bacterial plasmids mainly transfer via conjugation mechanism, however other mobile genetic elements such as transposons can also be transferred via conjugation (Huddleston, 2014; Thomas and Nielsen, 2005).

Transformation: In this process, bacteria acquire the free DNA from environment and incorporate them into their own genome. In brief, bacteria take up the free DNA from the environment which has been released by another bacterium. This DNA is then incorporated into their genome either by homologous or illegitimate recombination and finally, the newly incorporated DNA will be expressed in the recipient cell (Huddleston, 2014).

Transduction: In this process, antibiotic resistance genes are transferred via bacteriophages. During the replication process, bacteriophages accidently acquire host or donor genomic DNA. Once, the bacteriophage lyses the host cell they are released into the environment. These new phages then infect new bacterial host and transfer their acquired genome into the new recipient bacteria (Arber, 2014; Huddleston, 2014).

Bacterial resistance can be classified into several mechanisms which broadly fall into three groups - inactivation of antibiotic, altering or modification of antibiotic target and minimizing intracellular concentration of antibiotics (Blair et al., 2015; Dever and Dermody, 1991; Wright, 2011).

Inactivation of antibiotic: Antibiotics can be inactivated by bacterial enzymatic hydrolysis or modification (Blair et al., 2015; Džidić et al., 2008; Kong et al., 2010). β-lactamases are enzymes that many Gram-negative bacteria produce which can degrade β-lactam drugs. These enzymes hydrolyze the β-lactam ring which is a functional unit of this group of antibiotics thus rendering the antibiotics inactive (Blair et al., 2015; Bonnet, 2004; Kong et al.,

2010; Walsh, 2000; Wright, 2011). Antibiotics can also be inactivated by transferring a chemical group to their vulnerable sites by bacterial enzymes which prevents the antibiotics from binding to its target protein (Blair et al., 2015; Džidić et al., 2008; Wright, 2011) A number of different chemical groups such as adenyl, phosphoryl, or acetyl can be transferred to the antibiotic site by antimicrobial resistant enzymes transferase and thus modified the antibiotic (Blair et al., 2015; Džidić et al., 2008; Wright, 2011). Aminoglycosides, chloramphenicol, streptogramin, macrolides and rifampicin can be inactivated by these group transfer process (Džidić et al., 2008).

Altering or modification of antibiotic target: Target site modification can inhibit antibiotic function by making them unable to bind properly (Walsh, 2000). Mutational changes in bacterial structure can prevent the antibiotic from binding properly while retaining cellular functions. β-lactam, aminoglycosides, tetracyclines, fluoroquinolones, macrolides etc. are some of the drugs which can be inhibited by target alteration (Blair et al., 2015; Džidić et al., 2008; Mcdermott et al., 2003). For example, β-lactam drugs target bacterial peptidoglycan layer, however, mutation in bacterial PBPs can result in lower affinity to β-lactam drugs and thus confer resistance to them (Džidić et al., 2008).

Minimizing intracellular concentration of antibiotics: Active efflux pumps and outer membrane permeability of bacteria can minimize the antibiotic concentration into the cell and thus confer resistance (Džidić et al., 2008; Wright, 2011). Resistance to many antibiotics such as tetracyclines, chloramphenicol, macrolides, fluoroquinolones andaminoglycosides occurs in this way (Dever and Dermody, 1991; Mcdermott et al., 2003; Wright, 2010). For example, *tet*A, *tet*B and *tet*C are the genes which can encode efflux pumps which reduce the concentration of tetracycline within bacterial cells conferring resistance. Changes or down-regulation in porin

expression can affect the ability of antibiotics to enter the bacterial cell. β-lactams, chloramphenicol, and fluoroquinolones are the drugs which use porin channel to enter into the Gram-negative bacteria, changes in porin expression can therefore inhibit these antibiotics (Džidić et al., 2008).

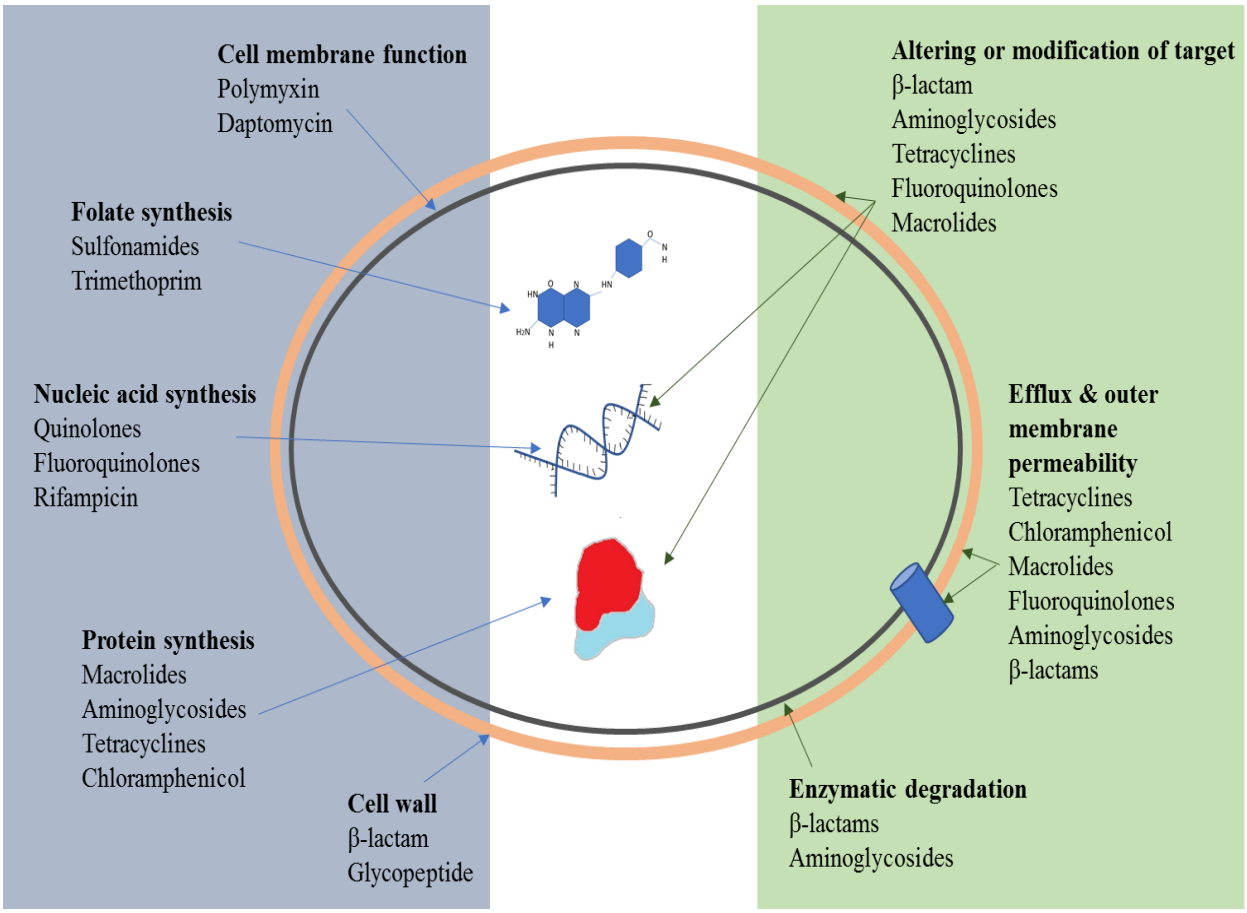

Figure 1.1: Antimicrobial targets and mechanism of antimicrobial resistance. Antimicrobial targets are highlighted on the left (grey) and mechanisms of resistance are listed on the right (green). Figure adapted from (Wright, 2010).

1.1.3 Antimicrobial resistance in Gram-negative bacteria

Antibiotic resistance is a complex and long standing problem in both Gram-positive and Gram-negative bacteria, however, resistance in Gram-negative bacteria is currently the most pressing issue (Kuenzli, 2016; Laxminarayan et al., 2013; Paterson, 2006). In Gram-negative bacteria, broad-spectrum β-lactamase resistance has increased globally due to indiscriminate antimicrobial use in humans and animals. Extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae are a major concern as these enzymes confer resistance to some important class of drugs such as penicillin, amoxicillin, 3rd generation cephalosporin (3GC) (Dahms et al., 2015; Kuenzli, 2016; Overdevest et al., 2011; Rubin and Pitout, 2014). A study found 1.7 times higher hospital cost for urinary tract infection caused by ESBL producing *E. coli* and *Klebsiella sp* comparing with other causal agents (Lee et al., 2006). Besides these, multi-drug resistance (MDR) is another threat for Gram-negative bacteria which limits the ability for clinicians to treat community onset infections (Vasoo et al., 2015). Besides health care related infections, ESBL producing Enterobacteriaceae were identified from community acquired infections (Kuenzli, 2016). Recently carbapenem-resistant Enterobacteriaceae including those producing New Delhi metallo β lactamase (NDM) and *Klebsiella pneumoniae* carbapenemase (KPC) have emerged which confer resistance to these drugs of last resort. Increased patient mortality rates are associated with carbapenemase producing Enterobacteriaceae (Vasoo et al., 2015).

1.1.4 Broad-spectrum β-lactamases

β-lactam antibiotics are important drugs in treating infections caused by many common bacterial pathogens including *E. coli*. In 2009, total antibiotic sales were 42 billion US dollars globally (Hamad, 2010). Antibiotics of β-lactam family contribute 65% to the total antibiotic

market worldwide (Thakuria and Lahon, 2013). This class consists of penicillins, cephalosporins, carbapenems, cephamycins, and monobactams (Holten and Onusko, 2000). β-lactam antibiotics bind with the PBPs of bacterial cell membrane and thus inhibit the cell wall synthesis. β-lactamases hydrolyse the β-lactam ring, thus inactivate these antibiotics (Džidić et al., 2008; Livermore and Woodford, 2006; Mcdermott et al., 2003; Paterson, 2006; Tenover, 2006; Walsh, 2000; Wright, 2011). Genes encoding β-lactamase enzymes can be found either chromosomally or on mobile genetic elements which includes plasmids, gene cassettes, or transposons (Babic et al., 2006; Drawz and Bonomo, 2010; Wright, 2005). β-lactamases are a broad group of enzymes have been classified using two schemes - (a) Ambler molecular classification and (b) Bush-Jacoby-Medieros functional classification. In the Ambler classification system, β-lactamases are grouped according to protein sequence similarity. There are four classes (A - D) in the Ambler scheme where class A, C and D are serine β-lactamases and class B are metallo β-lactamases (Ambler et al., 1991; Bush et al., 1995; Paterson and Bonomo, 2005). Conversely, functional similarities (substrate and inhibitor profile) of the enzymes are the basis of Bush-Jacoby-Medieros classification (Bush and Jacoby, 2010). Classification of β-lactamase enzyme is summarized in **Table 1.1**. Extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases and carbapenemases are the emerging enzymes in Enterobacteriaceae which are increasingly problematic (Pitout, 2012a).

Extended-spectrum β-lactamases (ESBLs): ESBLs are class A enzymes according to the Ambler classification. They can degrade broad-spectrum β-lactams which include penicillins, cephalosporins, and monobactams but not the cephamycins or carbapenems. These enzymes can be inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam or sulbactam (Chong et al., 2011; Paterson and Bonomo, 2005). SHV (except SHV-1), TEM (except TEM- 1, 2 and

13) and CTX-Ms are examples of some of the most common ESBLs presently. The parent enzymes SHV-1 and TEM-1, 2 and 13 can hydrolyze penicillin and first generation cephalosporins and be inhibited by clavulanic acid, but due to their narrow spectrum of activity are not considered to be ESBLs. Later, a number mutant of these parent enzymes has been discovered which having broad spectrum of activity are considered as SHV and TEM type ESBLs (Paterson and Bonomo, 2005; Rubin and Pitout, 2014). As of March 2017, 193 SHV and 223 TEM variants have been described (http://www.lahey.org). The CTX-M enzymes are a new family of ESBLs whose broad spectrum did not arise as a result of mutation from existing enzymes like TEM and SHV ESBLs, rather these were acquired by horizontal gene transfer from *Kluyvera sp* (Pitout, 2012a; Rubin and Pitout, 2014). The name CTX-M refers to the preferential hydrolytic activity of the enzymes against cefotaxime than ceftazidime, and "M" refers to Munchen where these enzymes were first described (Paterson and Bonomo, 2005; Rubin and Pitout, 2014). The number of CTX-M variants is increasing and as of March 2017, 172 variants of CTX-M were identified worldwide (http://www.lahey.org). The CTX-M enzymes can be classified into phylogenetic subgroups CTX-M-1, CTX-M-3, CTX-M-8, CTX-M-9, CTX-M-15 and CTX-M-25 based on clustering of their amino acid sequences (Bonnet, 2004; Hawkey and Jones, 2009).

Plasmid-mediated AmpC β-lactamases: This group of enzymes is class C based on Ambler classification, and group 1 according to the Bush-Jacoby-Medieros system (Jacoby, 2009). Although AmpC enzymes are structurally very similar to class A β-lactamases, they differ in that class C enzymes have more open binding sites for cephalosporins (Jacoby, 2009). These enzymes confer resistance to a broad spectrum of antibiotics which includes penicillin, cephalosporins, monobactams and cephamycins (Jacoby, 2009). Cloxacillin, oxacillin and

azetronam are good inhibitors of AmpC enzymes whereas these enzymes are poorly be inhibited by Class A enzymes inhibitors such as clavulanic acid, sulbactam and tazobactam (Jacoby, 2009). AmpC β-lactamase was first detected enzyme from *E. coli* which were resistant to penicillin while plasmid-mediated AmpC (pAmpC) genes were reported from *Enterobacter cloacae* in 1980s (Philippon et al., 2002). CMY, ACT, FOX, ACT, and DHA types are some of the examples of AmpC β-lactamases which derived from chromosomally encoded AmpC cephalosporinases of bacteria such as *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Aeromonas* spp and *Hafnia alvei* (Rubin and Pitout, 2014). The Lahey database is a curated list of β-lactamase genes, as of March 2017 a total 136 variants of CMY have been described (http://www.lahey.org).

Carbapenemases: Carbapenemases are a group of enzyme which are capable of hydrolyzing all classes of β-lactam drugs including carbapenems which are considered to be the last resort antimicrobial drugs (Nordmann et al., 2011). Carbapenemases fall into two major molecular families; metallo-β-lactamases and serine-β-lactamases (Queenan and Bush, 2007). Metallo β-lactamases (MBLs) differ structurally from the other β-lactamases by their requirement for at least one zinc atom at the active site which facilitates them to hydrolysis β-lactam drugs (Queenan and Bush, 2007). MBLs can be inhibited by metal ion chelators, such as EDTA and dipicolinic acid (Laraki et al., 1999), but are uninhibited by clavulanic acid, tazobactam or sulbactam. MBLs are Ambler class B and functional group 3 (Queenan and Bush, 2007). Verona integron encoded metallo β-lactamase (VIM), Imipenemase (IMP) and recently, New Delhi metallo β-lactamase (NDM) are the most common types of MBLs (Nordmann et al., 2011). Unlike MBLs, serine β-lactamases contain a serine at their active site. Metal ion chelators like EDTA do not inhibit these enzymes (Queenan and Bush, 2007). Serine β-lactamases belong

to Ambler class A and D. A variety of enzymes have been described within class A carbapenemases, some of them are chromosome-mediated such as SME, NMC-A, IMI-1, while some are plasmid mediated such as the KPC, IMI-2, GES (Queenan and Bush, 2007). In fact, plasmid-mediated KPC is the most commonly identified class A carbapenemase worldwide (Nordmann et al., 2011). Oxacillinases (OXA) with carbapenemase activity categorized as class D enzymes, have been extensively reported throughout the world (Nordmann et al., 2011). The OXA enzymes are a diverse family of β-lactamases including OXA-48, OXA-181 (Nordmann et al., 2011; Queenan and Bush, 2007).

Table 1.1: Classification of β-lactamases enzymes (Bush and Jacoby, 2010; Kanj and Kanafani, 2011; Rubin and Pitout, 2014)

Ambler Classification	Bush-Jacoby-Medeiros Group	Active site	Enzyme	Examples	Spectrum of resistance	Inhibitors
A	2be, 2br, 2c, 2e, 2f	Serine	ESBLs	TEM (except TEM-1, 2 and 13), SHV (except SHV-1), CTX-M	Penicillin, cephalosporin, Monobactam	Clavulanic acid, tazobactam, sulbactam (except 2br)
			Carbapenemases (KPC type)	KPC	Penicillin, cephalosporin, monobactam, meropenem cephamycin	
B	3a	Zinc-binding thiol group	Carbapenemases (Metallo β-lactamases)	NDM, VIM, IMP	All β-lactams	EDTA and other metal chelators
C	1	Serine	AmpC	CMY-2, FOX, ACT,	Penicillin, cephalosporin, cephamycin, monobactam	Cloxacillin, boronic acid
D	2df	Serine	Oxacillinase	OXA-1, OXA-48	Penicillin, carbapenem	NaCl

1.1.5 Integrons in Gram-negative Bacteria

In Gram-negative bacteria, integrons play an important role in horizontal resistance gene transfer between bacteria. Integrons are genetic element structured with three distinct components: integrase gene (intI), gene cassette receptor site (attI) and a promoter (Pc) which can recognize and carry gene cassettes on it (Hall and Collis, 1995). This integrase gene (intI) encodes integrase enzyme which facilitates the site specific insertion of gene cassettes on the gene cassette receptor site (attI) and once cassettes are inserted at attI, promoter expresses the gene cassettes associated gene (Michael et al., 2004). Integrons are not mobile themselves, but the gene cassettes within them can be independently mobilized (Fluit and Schmitz, 2004). Gene cassettes are small elements which can be incorporated into an integron or exist as a free, circular DNA molecules (Bennett, 1999; Hall and Collis, 1995). The gene cassette is a non-replicating mobile genetic element lacking its own promoter and is therefore expressed under the influence of the promoter within the integron. During the integration process, the attI of integron and attC of gene cassette consists of a core sequence where the site-specific recombination takes place. Gene cassette expression level varies with distance from the promoter; those near the promoter are expressed more strongly than cassettes more distant to the promoter (Bennett, 1999). Integrons may carry multiple gene cassettes, up to 8, resulting in multidrug resistance (Rowe-Magnus and Mazel, 2002). Integrons are categorized into three distinct classes: class 1, class 2 and class 3. Class 1 integrons are the most frequently identified integrons from various clinical isolates (Bennett, 1999; Fluit and Schmitz, 2004). The class 1 integron consists of 5’ and 3’ conserved regions (CR) separated by variable region. Gene cassettes are found within the variable region (Bennett, 1999). The essential features of class I integron like intI, attI and promoter all accommodated in the 5’ CR, while 3’ CR consists of genes sul1, which encodes

resistance to sulphonamides, a truncated qacΔE1, that has been shown to increase the minimum inhibitory concentration of benzalkonium chloride (Bennett, 1999). A variety of gene cassettes are associated with class 1 integrons, however, aadA gene cassette is most common (Fluit and Schmitz, 2004). Class 1 integrons containing gene cassettes has been identified to be frequent encoder of streptomycin-spectinomycin, trimethoprim and β-lactam resistance (Deng et al., 2015; Sandvang, 1999). These integrons are commonly located on plasmids which mediate rapid transfer to other organisms via conjugation (Fluit and Schmitz, 2004).

1.2 Epidemiology of antimicrobial resistant *Escherichia coli* in humans and animals in Canada

1.2.1 Broad-spectrum β-lactamases in human isolates

Drugs within the β-lactam family are the treatment of choice for treating a wide variety of community onset bacterial infections caused by Gram-negative bacteria (Shaikh et al., 2015). However, the emergence of resistance towards these drugs limits the clinicians ability to use existing drugs effectively. Organisms producing "newer β-lactamases" including the ESBLs, plasmid-mediated AmpC and carbapenemases are increasingly encountered in community settings throughout the world including Canada (Pitout, 2012a). ESBLs are most often associated with *E. coli, K. pneumoniae* and other members of the Enterobacteriaceae family. Following SHV and TEM type ESBLs, the CTX-M type enzymes have gained prominence throughout the world since 2000's (Pitout, 2012a). Among the diversity of variants of CTX-M identified from clinical isolates, CTX-M-14 and CTX-M-15 are the most prevalent around the world (Pitout, 2012a). In Canada, CTX-M-15 were identified as most predominant ESBL from hospital patients

(Baudry et al., 2008; Peirano et al., 2012). Multi-locus sequencing typing has identified strain associations of *E. coli* with CTX-M type ESBLs production. *E. coli* ST131 and to a lesser extent ST10 clonal complex which were found to be associated with the production of a most widespread and prevalent CTX-M-15 globally (Pitout, 2012a). Like the ESBLs, plasmid-mediated AmpC also were reported in *E. coli* from community onset urinary tract infection (UTI) (Pitout, 2012a). CMY-2 is the most commonly encountered AmpC type β-lactamase among the Enterobacteriaceae globally (Pitout, 2012a). In Canada, a study conducted in the Calgary Health Region, found that the CMY-2 type enzyme was the most prevalent type AmpC β-lactamases in *E. coli* from patients with UTI (Pitout, 2012a). In Canadian hospitals, surveillance studies have shown that 13.5% of 232 cefoxitin resistant isolates possessed CMY-2 (Mulvey et al., 2005). Since the mid 2000s, the emergence of carbapenemase producers has been described in Canada. Though low percentage of carbapenem-resistant Enterobacteriaceae (CRE) and carbapenem-resistant organisms (CRO) were reported in Canadian hospital patients in the year 2010 - 2014, however, there was an increasing trend of CRE (Public Health Agency of Canada, 2016). KPC was mostly encountered CRE followed by NDM-1 and Oxa-48 (Public Health Agency of Canada, 2016).

1.2.2 Broad-spectrum β-lactamases in agricultural animal isolates

Food producing animals are a potential source of antimicrobial resistance in community. Cattle, pigs and chickens are considered as major food animal species. There is a high possibility of bacterial contamination during slaughter process of these animals. Different countries have implemented surveillance programs to monitor antimicrobial resistance in healthy animals to assess the potential zoonotic risk. ESBL producing *E. coli* is a growing concern in food and food producing animals globally (Bhoomika et al., 2016). A variety of ESBLs including: CTX-M-1,

CTX-M-2, CTX-M-8, CTX-M-14 and CTX-M-15 have been identified from poultry in different regions while CTX-M-1 is the predominant ESBL in agricultural animals (Belmar Campos et al., 2014; Börjesson et al., 2013). A variety of pAmpC including CMY-2, DHA-1 and ACC-1 have been identified in *E. coli* from chickens, although CMY-2 is the most common (Börjesson et al., 2013; Reuland et al., 2014). In Canada, Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) is monitoring AMR in food producing animals in national level (Government of Canada, 2015a). CIPARS findings showed isolates from chicken were resistance towards 3GC and amoxillin-clavulanic acid, which suggests that Canadian chicken might harbor broad-spectrum β-lactamase genes (Government of Canada, 2015b). One study in Canada found CMY-2 as predominant β-lactamase type in chicken meat which possessed resistance to 3GC (Sheikh et al., 2012). Comparing with other part of the world, there is very limited data on β-lactamase producing organisms from agricultural animals in Canada.

1.2.3 Antimicrobial resistance studies in Canada

The Public Health Agency of Canada (PHAC) plays a major role in antimicrobial resistance surveillance studies in Canada. The PHAC select certain bacteria of high importance in their AMR studies from the list of 138 disease causing infectious pathogen which are posing resistant throughout the world (Public Health Agency of Canada, 2016). In health-care settings, PHAC surveillance systems currently monitor resistance in *Clostridium difficile*, carbapenem-resistant organisms (CRO) including carbapenem-resistant Enterobacteriaceae (CRE), methicillin-resistant *Staphylococcus aureus (*MRSA) and vancomycin-resistant *Enterococci* (VRE). In community settings, PHAC's surveillance systems monitors *Streptococcus pneumoniae*, Group A *Streptococcus* (GAS), *Neisseria gonorrhoeae* and *Mycobacterium tuberculosis* (Public Health Agency of Canada, 2016). PHAC also monitors zoonotic and

foodborne organisms including *E. coli*, *Salmonella* and *Campylobacter* from human clinical cases, retail food, animals at slaughter, on farm, and from veterinary diagnostic labs through CIPARS (Public Health Agency of Canada, 2016). CIPARS performs surveillance of antimicrobial use in agriculture and the presence of antimicrobial resistant organisms. They collect data of antimicrobial use in human and animal population, as well as select bacteria isolated from human and animal diagnostic submissions (Government of Canada, 2015a). Retail food samples, including fresh raw turkey, beef, pork, and chicken are included to provide exposure estimates close to the point of consumption (Government of Canada, 2015a). At the abattoir level, caeca of beef cattle, broiler chickens and pigs are sampled in order to reflect transmission of monitored antimicrobial resistance through the food chain prior to entry into the retail segment (Government of Canada, 2015a). On-farm surveillance monitors microbial resistance in bacteria from fecal samples from grower-finisher pigs and broiler chickens to provide surveillance data at the production level and to enable estimation of associations between husbandry practices, including antimicrobial use, and antimicrobial resistance (Government of Canada, 2015a). Data generated from animal, food and human isolates are analyzed to identify temporal changes in antimicrobial resistance and use annually (Government of Canada, 2015a).

1.3 The Canadian chicken industry

1.3.1 The quota system and production flows

In Canada, the poultry industry is well regulated by national bodies and provincial level organizations to ensure Canadians have access to safe poultry products. In Ontario, the Ministry

of Agriculture, Food Safety and Rural Affairs (OMAFRA), Chicken Farmers of Canada (CFC), Chicken Farmers of Ontario (CFO) are the key regulatory organizations. OMAFRA is responsible for regulating provincially inspected slaughter facilities. CFC is the national regulatory body for chicken industry in Canada. CFC determines the national supply of chicken based on the Canadian market requirements. CFC also determines the provincial production rate based on the provincial commodity groups demand and market requirements (Chicken Farmers of Canada, 2016a). CFO determine the number of allocation demand within the province to the national agency after consulting with industry stakeholders (OMAFRA, 2014). Ontario's quota system is part of the supply managed system across Canada for the broiler chicken industry. Quota is a license issued by CFO to produce and market chicken in the province. It limits the farmer to produce and market a certain amount of chicken meat within a specific time period set by CFO. A unit of quota is equal to 13 kilograms of chicken production per annum, and quota holding farmers must have at least 14,000 units of quota equating to approximately 182,000 kilograms of chicken production per year (Chicken Farmers of Ontario, 2016a). In 2015, there were 36,450,014 quota units in Ontario representing approximately 489 million kilos of chicken. Until 2014, farmers without quota were permitted to produce no more than 300 chickens per year in Ontario. Non-quota holding farms are often described as backyard or small flock producers (Chicken Farmers of Ontario, 2016a).

1.3.2 Overview of small scale vs. industrial production

Canada's poultry industry is growing and it produced chicken products that were worth $2.4 billion in 2015 and contributed $6.8 billion to Canada's Gross Domestic Product (Chicken Farmers of Canada, 2016b; Government of Canada, 2016a). There were 2690 regulated chicken producers in Canada which produced 1.1 billion kilograms of chicken in 2015 (Government of

Canada, 2016a). The per person consumption of chicken meat has increased in 2015, while pork and beef consumption have declined (Government of Canada, 2016b). Canada also exported a small number of chicks (5.8 million) to 9 different countries and 133.2 million kilograms of chicken meat products to 63 different countries in 2015 (Government of Canada, 2016b). The United States is the largest export market for Canadian chicken. As Canada's most populated province with a population of 13.9 million (Government of Canada, 2016c), Ontario is also the largest chicken producer in Canada with 1,155 chicken farmers and 42 processors, the Ontario chicken industry contributed $2.2 billion to Canada's GDP (Chicken Farmers of Canada, 2016b). Besides large-scale commercial farming, there is an increased demand for local or small-scale chicken in Canada. In prior regulation, non-quota holding farmers were not allowed to have more than 300 birds annually. However, to meet the local demands, Canadian provinces have launched different schemes which will allow small, independent, locally based farmers to have their small-scale chicken farms. In Ontario, artisanal chicken program is one of them which will allow the farmers to grow between 600 to 3000 chickens per year without holding any quota. Until June 2016, there were total 103 artisanal chicken farmers with average 1564 chickens per farm (Chicken Farmers of Ontario, 2016b).

1.3.3 Provincially vs. federally regulated abattoirs

There are two regulatory schemes for abattoir inspection in Canada, federal and provincial. Federally inspected abattoirs have a large slaughter capacity and process approximately 95% of food producing animals (Government of Canada, 2015a). Meats for inter-provincial consumption or export must have to pass though federally inspected abattoirs. The meat inspection of federally inspected abattoirs is performed by Canadian Food Inspection Agency (CFIA) (Government of Canada, 2013). In Ontario, most of the large commercial broiler

chickens are slaughtered in federally inspected abattoirs, while smallholder or "backyard" flocks typically raised by small-scale producers are slaughtered in provincially inspected abattoirs. Current resistance surveillance done by CIPARS focuses only on birds passing through federally inspected abattoirs, birds processed in provincially inspected abattoirs are not represented in CIPARS sampling (OMAFRA, 2016).

1.4 Zoonotic and foodborne transmission of *Escherichia coli* from chickens into people

Zoonosis is a term used to describe those diseases which transmitted to human from animals. Those pathogens transferred by contaminated food are sub-classified as foodborne. Most *E. coli* strains are colonizers which are part of the gastrointestinal microbiota of healthy individuals (Canny and McCormick, 2008). There are some opportunistic and pathogenic *E. coli* which can cause a variety of illnesses in healthy human and animals (Kaper et al., 2004). *E. coli* are often grouped into one of six pathotype categories: enteropathogenic, enterotoxigenic, enterohemorrhagic, enteroinvasive, enteroaggregative and diffuse-adhering. While extra-intestinal pathogenic *E. coli* (ExPEC) can be categorized as uropathogenic and meningitis associated (Hammerum and Heuer, 2009; Kaper et al., 2004; Manges and Johnson, 2012). Extra-intestinal pathogenic *E. coli* (ExPEC) are responsible for most community and hospital onset human infections such as urinary tract and bloodstream infections, sepsis (Manges and Johnson, 2012). Avian pathogenic *E. coli* is associated with extra-intestinal infections in chickens, turkey and other poultry species including pericarditis, airsaculitis, polyserositis and septicemia (Dho-Moulin and Fairbrother, 1999; Kaper et al., 2004). Genes coding for virulence factors of these pathogenic strains are often located in the chromosome on pathogenicity islands (PAI) which are

large genomic regions not found in commensals. Interestingly, these pathogenic strains can acquire additional factors which are encoded by mobile genetic elements such as plasmids, bacteriophages and transposons, thus can be transmitted to other bacteria easily (Kaper et al., 2004). Food animals and retail meat products are a potential reservoir for human ExPEC infection. Compared with beef or pork, chicken meat has been found to be a source of ExEPC which are more genetically similar to isolates causing UTIs in human (Bergeron et al., 2012; Manges and Johnson, 2012). Different studies have found an association between antimicrobial use and the presence of resistance in animals (Agersø et al., 2012; Cohen Stuart et al., 2012; Lowrance et al., 2007). A significant relationship between incidence of ceftiofur-resistant *Salmonella enterica* serovar Heidelberg in humans and retail chickens and the use of ceftiofur in chicken was identified in Canada demonstrating the link between animals and people and highlighting the role of chickens specifically (Dutil et al., 2010). Similarly, in the Netherlands, the predominant indistinguishable ESBL genes have been found in chicken and human rectal swabs specimen (Overdevest et al., 2011). Therefore, we argue that chicken meat a plausible source of resistance in humans.

1.5 Using *Escherichia coli* as a resistance surveillance target

1.5.1 Strategies for representative sampling

Resistance surveillance programs around the world such as CIPARS in Canada, NARMS in USA, DANMAP in Denmark, JVARM in Japan have targeted *E. coli* because of its usefulness as an indicator organism for antimicrobial resistance in other taxa of the gut microbiota (Franklin et al., 2001; Government of Canada, 2015a; Morrison and Rubin, 2015; Zhao et al., 2012).

Furthermore, the relative case of *E. coli* recovery (97-100%) from chicken caeca makes it an ideal target for resistance surveillance compared to *Salmonella* or *Campylobacter* for which recovery rates (13-28%) are much lower (Government of Canada, 2015b). Testing *E. coli* is therefore an efficient means to an estimate of resistance prevalence. Furthermore *E. coli* can be zoonotic and antimicrobial resistant *E. coli* isolates colonizing and infecting people have been shown to be an important food borne zoonoses which may result in urinary tract and bloodstream infections therefore having a direct impact on human health (Bergeron et al., 2012; Kluytmans et al., 2013; Vincent et al., 2010). Therefore, *E. coli* could be a good choice of organism for starting resistant surveillance in chicken population.

1.5.2 Sample collection and processing strategies

Effective resistance surveillance requires the development of sampling strategies which balance the relationship between cost effectiveness, feasibility, and statistical plausibility (Benedict et al., 2013; Yamamoto et al., 2014). The 'single sample per farm' strategy recommended by European Food Safety Authority (EFSA) has been found to be the most sensitive sampling strategy for detecting resistant organism at the population level (Yamamoto et al., 2014). This strategy has been used by surveillance programs in different countries including Canada, Japan, Sweden, Denmark and Netherlands (Yamamoto et al., 2014). Which materials should be targeted as samples depend on several factors such as whether the study targets retail meat, abattoirs or animals on farm. Collecting caeca content will be a good choice as it helps to avoid misinterpretation related to cross-contamination and to better reflect antimicrobial resistance in bacteria that originated on the farm (Government of Canada, 2015a). In the majority of chicken microbiota studies, ceca used as a sample for its productivity, health and wellbeing of chicken, however, it requires sacrificing the birds (Stanley et al., 2015). While fecal sampling is

more convenient comparing with cecal content as it allows using the same bird for repeated sampling without killing (Stanley et al., 2015). However, fecal samples are susceptible for environmental contamination.

1.5.2.1 Culture and identification of Escherichia coli

Propagation and identification of *E. coli* can be done by using bacterial culture media and different biochemical tests. MacConkey agar is commonly used for the isolation of *E. coli* in clinical laboratory settings. MacConkey agar contains lactose as fermentable sugar and neutral red as pH indicator. It also contains bile salts and crystal violet as inhibitors which suppress the growth of Gram-positive bacteria. On MacConkey agar *E. coli* produce deep red colonies as the organism is lactose-positive, and fermentation of this sugar will cause the medium's pH to drop, leading to darkening of the medium (Markey et al., 2013). Eosin methylene blue (EMB) agar is occasionally used in diagnostic laboratories for detection of *E. coli*. This agar contains eosin and methylene blue as pH indicator, sucrose and lactose as carbohydrate source. The pH indicator dyes turn into dark purple color at low pH and suppresses the growth of Gram-positive bacteria partially. Growth of *E. coli* on EMB agar produces black colonies with a greenish-black metallic sheen (Markey et al., 2013). Besides that, different selective media are used to detect specific bacterial strains such as CHROMagar ESBL which can be helpful to identify ESBL producing *E. coli*. Following a presumptive identification of *E. coli* from a differential media, the biochemical properties of an organism can be useful for identification. On **Table 1.2**, some important characteristics for differentiating members of Enterobacteriaceae family are included.

Table 1.2: Biochemical properties of clinically important members of Enterobacteriaceae family

	ID	MR	VP	Cit	Ure	PD	HS	Mot	GL
E. coli	+	+	_	_	_	_	_	+	_
K. pneumonia	_	_	+	+	+	_	_	_	_
S. enterica	_	+	_	+	_	_	_	+	_

ID - Indole production, MR - Methyl red, VP - Voges-Proskauer, Cit - Citrate, Ure - Urease, PD - Phenylalanine deaminase, HS - Hydrogen sulphide, Mot - Motility, GL - Gelatin liquefaction; Table adapted from (Markey et al., 2013).

1.5.3 Detection and characterization of antimicrobial resistance

1.5.3.1 Phenotypic antimicrobial susceptibility testing

The goal of antimicrobial susceptibility testing is to determine whether an isolate is resistant to drugs of interest. Commonly used susceptibility testing methods include disk diffusion, gradient strip, agar dilution and broth microdilution. Test results with minimum inhibitory concentration (MIC) value will give quantitative data where interpreting zone diameters will give categorical data. The disk diffusion test yields a categorical description while all others are quantitative (Jorgensen and Ferraro, 2009). Disk diffusion and broth microdilution are the two methods which are commonly used in veterinary medicine as well as resistance surveillance programs (Benedict et al., 2013; Brooks et al., 2003).

Disk diffusion testing: This method also known as Kirby-Bauer antibiotic testing was first described by Bauer, Kirby, Sherris and Turck in 1966 (Bauer et al., 1966). Antibiotic impregnated discs are placed on agar plates inoculated with the test bacterium. Antimicrobial agent will diffuse and create a zone of diffusion which will inhibit the growth of susceptible bacteria (Markey et al., 2013). Based on the size of the zone diameter, to the test can be interpreted using standardized guidelines to determine whether the organism is resistant or susceptible. The first step is to prepare a bacterial suspension in saline or deionized water. The inoculum turbidity must be matched with 0.5 McFarland standard, which corresponds to approximately 1.5×10^8 CFU/ml. A lawn of inoculum is streaked out evenly onto the surface of muller-hinton agar (MHA) plate. Typically, up to 12 antimicrobial disks can be placed on a single plate at a time. Following inoculation, plates are inverted and incubated at 35°C for 16-18 hours. The zones of bacterial growth inhibition around each drug are measured to the nearest millimeter using a ruler or slide-calipers (**Figure 1.2**), and the size of the zone of inhibition can

then be interpreted using Clinical and Laboratory Standards Institute (CLSI) or EUCAST criteria (Coyle, 2005; Jorgensen and Ferraro, 2009). This method is simple, inexpensive, allows for testing customized drug panels but only provides categorical data which is a key drawback (Coyle, 2005).

Gradient Strip Test: Gradient strips (such as E-tests) are commercially available thin inert non-porous plastic strips impregnated with predefined antimicrobial gradient underside and the concentration scale is on the surface of it (Jorgensen and Ferraro, 2009). In brief, bacterial inoculum preparation and plating on agar media as described above. Typically, 4-6 strips can be placed on to a 150-mm plate in a radical fashion. During this incubation period, there is an establishment of antimicrobial gradient concentration around the strip because of immediate release of the drug. Following overnight incubation, the bacterial growth is visible and the tests are read by viewing a tear drop shaped inhibitory zone along the strip (**Figure 1.3**). The MIC is determined where the lower part of the inhibitory zone intersects with the gradient strip (Jorgensen and Ferraro, 2009).

Broth microdilution: Broth microdilution is a widely used test to determine antimicrobial MIC (Coyle, 2005). In this method, test bacterium is inoculated on a series of tubes containing antibiotic on different concentration. The highest concentration which will inhibit the growth of an organism is defined as the MIC (Markey et al., 2013). Commercially prepared tests (such as Sensititre) are available in 96 well microtiter plate format contains multiple doubling dilutions of a variety of antimicrobial agents. Each plate also contains positive and negative controls (Coyle, 2005; Jorgensen and Ferraro, 2009). In this test, MICs are determined using a manual or automated viewing device by observing the lowest concentration of antimicrobial

agent which completely inhibits growth of the organism (**Figure 1.4**) (Coyle, 2005; Jorgensen and Ferraro, 2009).

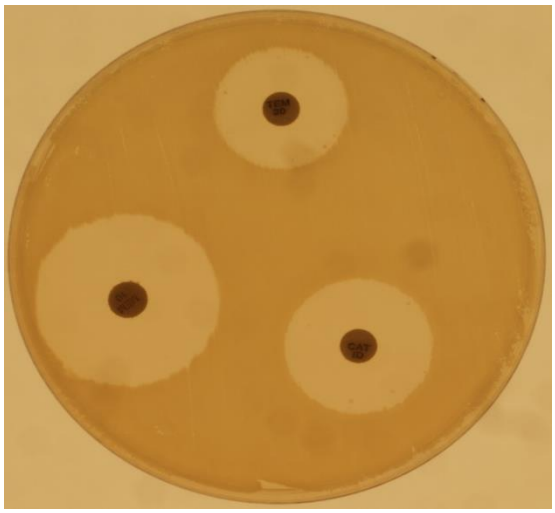

Figure 1.2: Kirby-Bauer disk diffusion test.

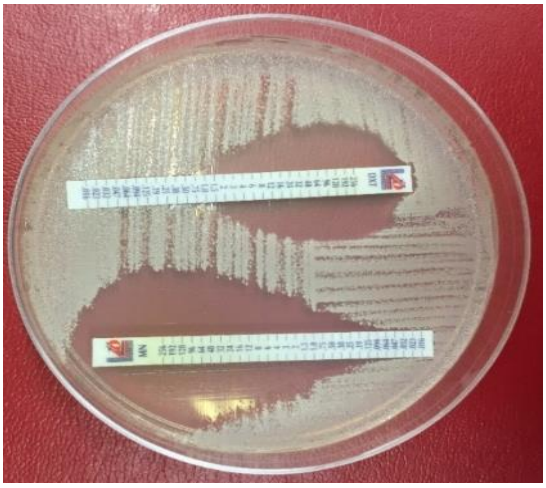

Figure 1.3: Antimicrobial gradient strips and tear drop shaped inhibitory zones.

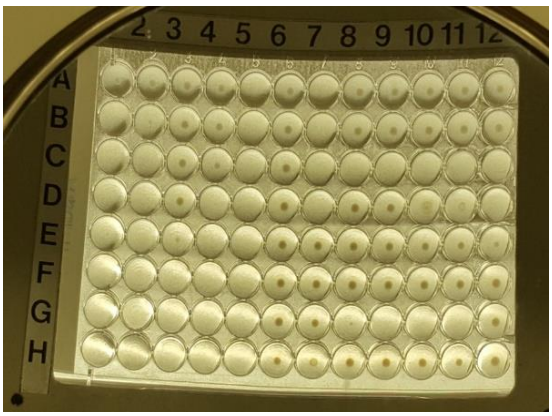

Figure 1.4: Broth micro-dilution antimicrobial susceptibility test.

1.5.3.2 Identification of resistance genes

The polymerase chain reaction (PCR) is a technique to amplify specific DNA sequences and can be applied to detect bacterial resistance genes. This technique produces millions of copies of target sequence of resistance gene DNA within a short time using a heat stable DNA polymerase (*Taq*) to mimic the natural process of DNA replication. *Taq* polymerase uses existing single-stranded DNA as a template and attaches dNTPs into the growing strand of DNA to make complimentary copy of DNA template (Bartlett and Stirling, 2003). Typically, PCR consists of 20 - 40 cycles of the three basic steps; denaturation, annealing and elongation (Viljoen et al., 2005). This technique can be applied to identify ESBL, AmpC or carbapenemase genes from resistant isolates in order to understand if there are epidemiologically resistant genes present.

Sequencing of PCR amplicons is commonly done to identify the allele of amplified loci and identify polymorphisms. The dideoxy method of DNA sequencing (Sanger sequencing) is often employed for amplicon sequencing. This method utilizes nucleotide triphosphates called dideoxynucleoside triphosphates (ddNTP) which substitute for deoxynucleotides at random positions during polymerization resulting in termination of DNA synthesis in a base-specific manner. Four PCR reactions are performed where each contains one dideoxynucleoside triphosphate (ddNTP) analog in addition to the four nucleotides (ATP, CTP, GTP or TTP) to terminate at A, G, C and T residues (Sanger et al., 1977). Each of these four sets will create fragments which will be resolved by electrophoresis in four separate lanes or can be visualized fluorescently using labeled ddNTP.

1.5.4 Molecular epidemiological techniques

1.5.4.1 Pulsed-field gel electrophoresis (PFGE)

Until the introduction of whole genome sequencing, pulsed-field gel electrophoresis (PFGE) has considered as the gold standard for comparing bacterial relatedness (Goering, 2010; Jordan and Dalmasso, 2015). PFGE is a molecular epidemiological tool used to identify and characterize outbreaks of food-borne disease or antimicrobial resistant organisms. PFGE is versatile, reproducible, relatively inexpensive, highly discriminatory and results in good epidemiological concordance for many pathogens (Jordan and Dalmasso, 2015). This method was first used for yeast *Saccharomyces cerevisiae* in 1980's for genome mapping by separating the large DNA fragments (Schwartz and Cantor, 1984). By the mid 1990's, PFGE was employed in surveillance studies to detection and identify disease clusters (Jordan and Dalmasso, 2015). In 1996, the US Center for Disease Control and Prevention (CDC) introduced PulseNet, a laboratory network utilizing PFGE based DNA fingerprinting targeting food-borne pathogens. Currently, 82 countries participate in the PulseNet system. PFGE is limited by the requirement for specialized laboratory equipment and trained staff, time consuming, less discriminating ability than whole genome sequencing (CDC, 2016a). In brief, this method involves embedding the bacterial DNA in agarose plug, digestion with restriction enzymes and resolution of fragments to reveal banding patterns. Banding pattern are analyzed using computer assisted analysis of digitized images. Isolates can be defined as indistinguishable, closely related, possibly related and unrelated based on their independent genetic event and number of fragment differences. Isolates with no fragment differences would be called indistinguishable while those differing by 2-3 bands is closely related. Isolates with 4-6 band differences are possibly related. If less then 50% of the isolates bands match with outbreak strain then that isolates are considered

as non-related strain (Tenover et al., 1995). Groups of isolates with 80% or greater relatedness can be defined as clusters which corresponds to the criteria of the “possibly related” (4 to 6 bands difference) (Peirano et al., 2012).

1.5.4.2 Multi-locus sequence typing (MLST)

MLST is a nucleotide sequence based technique used for unambiguous characterization of bacteria. MLST schemes have been developed for a variety of bacteria to identify clonal groups and large-scale phylogenetic relatedness. This technique targets multiple protein coding housekeeping genes found in all isolates of a particular species (Wirth et al., 2006). Because housekeeping genes serve metabolic functions, they are under stabilizing selection pressure; changes in the amino acid sequence of these proteins occur slowly and therefore provide reliable insight into the relatedness of isolates (Bergeron et al., 2012; Johnson and Russo, 2005; Maiden et al., 1998). Most MLST schemes include 300-700 bp regions of seven housekeeping genes from the core genome are selected, amplified and sequenced (Cooper and Fel, 2004). The sequence of gene is given a distinct allele number using a centralized database, unique allele numbers are assigned whether they differ by a single nucleotide or by multiple polymorphisms (Urwin and Maiden, 2003). The alleles of each of the seven housing keeping genes of each isolates are combined into an allelic profile and assigned a sequence type (ST). Sequence types can be grouped together into clonal complexes comprising closely related strains. MLST is a good typing technique comparing with other typing methods as it can detect the changes on nucleotide sequences of bacterial DNA, highly reproducible, does not require any sophisticated instruments or expensive reagents, it’s a conventional PCR based techniques. Most importantly, MLST data are analyzed based on centralized database yielding portable data allowing

comparisons the ST of an individual isolate to the global database (Belén et al., 2009; Wirth et al., 2006).

Objectives

- To determine if broad spectrum β-lactamase genes are present in *E. coli* from chickens raised in small flocks in Ontario, Canada.
- To determine the relatedness and sequence types of *E. coli* isolates from chickens raised in small-scale flocks.
- To compare the use of selective and non-selective media for the detection of β-lactamase producing *E. coli* in chickens coming from Canadian large commercial flocks.

2 ESBL and AmpC-β lactamase producing *Escherichia coli* isolates from chickens raised in small flocks in Ontario, Canada

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Author contribution:

Conceived and designed the experiments: JER, SAM, RJR, MAR. Sample collection and preparation: LAL. Performed the experiments: KKG. Analyzed the data: KKG, JER. Wrote the paper: KKG.

2.1 Abstract

Meat producing animals including chicken play an important role in dissemination of antimicrobial resistant bacteria to the community. In Canada, there is a lack of data about the prevalence of antimicrobial resistance in bacteria from chickens raised by small producers and passing through provincially inspected abattoirs. Therefore, this study was conducted to detect the presence of broad-spectrum β-lactamase producing *E. coli* and compare their relatedness with susceptible strains from chickens raised in small flocks in Ontario. A total of 1025 cecal samples (205 flocks, 5 birds each) were collected at provincially inspected abattoirs. A total of 99 3rd generation cephalosporin resistant isolates were identified by broth micro-dilution using Sensititre®. All these resistant isolates were screened for ESBL or AmpC β-lactamase by PCR. Finally, the 99 β-lactamase producers and 162 randomly selected susceptible *E. coli* isolates collected from the same 205 flocks were characterized by pulsed-field gel electrophoresis (PFGE) to determine their relatedness. To identify the sequence type, 24 isolates were characterized using MLST. A total of 55 ESBL producing strains were isolated from 26/205 (12.7%) of the flocks sampled while 39 AmpC β-lactamases were from 31 (15.1%) flocks. CTX-M-1 and CMY-2 were the most prevalent ESBL and AmpC enzyme respectively. While the majority of isolates were unrelated, 9 clusters (at least 3 isolates ≥85% similar) were identified by PFGE. Characterization of isolates by multi-locus sequence typing identified 16 different STs and 4 different ST clonal complexes. ST10 clonal complex was observed in both cluster and non-cluster forming isolates. Our results suggest that chickens raised in small flocks in Ontario may be a source of *E. coli* resistant to clinically relevant drugs, and that the dissemination of this resistance in chickens is most likely associated with the spread of plasmids rather than particular *E. coli* clones.

2.2 Introduction

Antimicrobial resistance is an emerging problem throughout the world including Canada. Particularly resistance associated with Gram-negative bacteria has increased globally (Hsu et al., 2010). Serious hospital acquired and community onset bacterial infections in humans including urinary tract infection are commonly caused by *E. coli* (Livermore, 1998; Paterson, 2006). In chickens, extra-intestinal infections with *E. coli* fall into syndrome "colibacilllosis" which describes a constellation of syndromes affecting chickens including airsacculitis, cellulitis, pericarditis, perihepatitis and respiratory distress (Kemmett et al., 2013). β-lactam antibiotics are important drugs in treating infections caused by *E. coli*, however, β-lactamase are the bacterial resistance enzymes which hydrolyse and make these antibiotics inactive (Livermore and Woodford, 2006).

Isolates of Enterobacteriaceae possessing ESBL and AmpC β-lactamases are increasingly common in human as well as animal population including chicken (Pitout, 2012b). Current data indicate that CTX-M β-lactamases are increasing and are the most prevalent ESBL type. In Europe chicken *E. coli* isolates have been extensively studied; TEM-52, CTX-M-14 and CTX-M-32 types ESBLs enzymes were detected in cefotaxime-resistant *E. coli* obtained from broilers in Portugal (Costa et al., 2009), while CTX-M-1, CTX-M-32 and SHV-12 types were found in Italy (Bortolaia et al., 2010). In Switzerland, CTX-M-1, SHV-12 and TEM-52 ESBLs were detected in *E. coli* strains recovered from faecal samples from healthy chickens (Geser et al., 2012a). The high prevalence of ESBLs and pAmpC producing *E. coli* in food producing animals has also been found to be common in Europe. In Canada, data from surveillance programs such as Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) findings

suggest that Canada may also have a high prevalence of broad-spectrum β-lactamase producing organisms.

A potential hazard resulting from the selection and spread of antimicrobial resistance in food animal production is the transmission of resistant bacteria or plasmids carrying resistance genes to humans via contact with animals or contaminated food (Sabaté et al., 2008). The relationship between in vitro resistance to β-lactams and ceftiofur usage has been observed in a number of food animal species including chicken (Agersø et al., 2012; Cohen Stuart et al., 2012; Lowrance et al., 2007). A positive correlation between the incidence of ceftiofur resistant organisms in human and in chickens and use of ceftiofur in hatcheries were observed in Canada (Dutil et al., 2010). These Canadian findings demonstrated the link between antimicrobial usage in chickens and resistant organisms in people (Dutil et al., 2010). This evidence suggests that chicken meat is a plausible source of resistance in humans in Canada.

In Canada, the CIPARS abattoir program, exclusively samples chickens processed in federally inspected abattoirs, which process the vast majority of food animals (Government of Canada, 2015a). These birds originate from large producers that supply uniform chickens (age, weight and breed) using standard husbandry. Birds which are slaughtered outside of the federal system and are processed in provincially inspected facilities are minor contributors to overall production in Canada (Government of Canada, 2016d). These birds may be heterogeneous in terms of age, weight and breed and include chickens raised on small, non-quota farms which until 2014 produced <300 birds/year. It should be noted that the increasing popularity of local food movements, farmer's market, and farm gate sales may result in an increase in the proportion of chickens slaughtered in provincial facilities over time. Because the microbiota changes with age, including changes in resistance and virulence in *E. coli*, chickens passing through

provincially inspected facilities may be colonized with *E. coli* populations with different resistance profiles than those captured by CIPARS (Kemmett et al., 2013; Ozaki et al., 2011). While the use of antimicrobials on any given chicken farm is ill-defined (Agunos et al., 2012), use by small producers who may have poorer bio-security and therefore have a higher burden of disease, house other animal species on unspecialized farms, or that have less consultation with poultry veterinarian is even less predictable. Therefore, although chickens from provincially inspected facilities account for a small percentage of the chicken consumed in Ontario, they may have a higher prevalence of resistant *E. coli* and therefore a disproportionately high impact on human health.

As the ESBLs are increasingly common among *E. coli* isolated from community settings (Woerther et al., 2013), determining the presence of these among chicken *E. coli* is important to better understand the epidemiology of resistance. Furthermore, finding resistant *E. coli* strain type in chicken will help to assess the risk of zoonotic transmission of resistance determinants. Overall the objective of this study to determine and characterize the broad-spectrum β-lactamase producing *E. coli* in chickens originating from small flocks passing through provincially inspected abattoirs in Southern Ontario, Canada.

2.3 Method and Materials

2.3.1 Study design

This study was conducted to identify broad-spectrum β-lactamase encoding *E. coli* and determine the clonal relationship of these isolates. As human resistant pandemic clone (*E. coli* ST131) has been identified health care settings, so we also investigated whether chickens from

Canadian small-scale flocks were harboring the same. This was a collaborative study with researchers at the University of Guelph. Sample collection, isolation and identification of bacteria and antimicrobial susceptibility testing was performed at the University of Guelph. *E. coli* isolates were sent to the University of Saskatchewan where additional molecular characterization was done.

2.3.2 Samples for Molecular characterization

Cecal samples were collected at slaughter from 1025 chickens originating from 205 small flocks (5/flock) at provincially inspected abattoirs in southern Ontario. Samples were selectively cultured for *E. coli* on MacConkey and CHROMagar ESBL, the antimicrobial susceptibility of isolates was determined by broth micro-dilution. All 3rd Generation Cephalosporin (3GC) resistant and a subset of susceptible isolates were sent on broth packed in cooling box to the University of Saskatchewan for molecular characterization.

2.3.3 Detection of ESBLs, AmpC β-lactamase and class I integrons using PCR

A total of 99 isolates resistant to 3GC were subjected to PCR to detect ESBL and AmpC encoding genes. Bacterial DNA was crudely extracted from the isolates by boiling (boil-prep). Previously published primers of CTX-M-U, CTX-M-G-1 and CMY-2 gene were used to detect ESBL and AmpC genes as well as class I integrons (**Table 2.1**). PCR reactions were carried out in 25µL volumes including 24µL of master-mix and 1µL of boil prep DNA template. In each set of reactions, positive and negative controls were included. Thermocycler conditions were as follows: initial denaturation at 94°C for 6 mins followed by 35 cycles of: denaturation at 94°C for 1 min, annealing at 61°C (CTX-M-U, CTX-M-G1)/68°C (CMY-2) for 1 min, elongation at

72°C for 1 min followed by final extension at 72°C for 10 mins. For class I integrons, touch-down PCR was performed with the following cycling conditions: 94°C for 6 mins, 22 cycles of 94°C for 1 min, 78°C -1°C for 1 min, 72°C for 1 min, 15 cycles of 94°C 1 min, 56°C -1°C for 1 min, 72°C for 1 min with a final extension at 72°C for 10 mins. Amplicons were resolved by electrophoresis using 1% agarose gel at 110 volts for 30 minutes. Gels were then visualized under UV light using the AlphaImager® HP (Fisher Scientific, ON, Canada).

Table 2.1: PCR primers used for detecting ESBL, AmpC β-lactamase and class I integron

Primer Name	Primer Sequence	References
CTXM-U	5’ -ATGTGCAGYACCAGTAARGTKATGGC- 3’	(Munday et al., 2004)
	5’ -TGGGTRAARTARGTSACCAGAAYCAGCGG- 3’	
CTXM-G1	5’ -GTTGTTAATTCGTCTCTTCC- 3’	(Pitout et al., 2004)
	5’ -AGTTTCCCCATTCCGTTTC- 3’	
CMY-2	5’ -ATGATGAAAAAATCGTTATGCTGC- 3’	(Kruger et al., 2004)
	5’ -GCTTTTCAAGAATGCGCCAGG- 3’	(Hasman et al., 2005)
Class I integron	5’ -GGCATCCAAGCAGCAAG- 3’	(Pellegrini et al., 2011)
	5’ -AAAGCAGACTTGACCTGA- 3’	

2.3.4 PCR amplicon purification and nucleotide sequencing

PCR products were purified using EZ-10 Spin Column PCR purification kit (Bio Basic Canada Inc., ON, Canada) and DNA was quantified by spectrophotometrically (NanoDrop™ 1000). Purified DNA with ~1.8 ratio of absorbance at 260 nm and 280 nm and 2.0 - 2.2 of 260/230 values were considered to have sufficiently high quality for sequencing. DNA solutions with a concentration of 10 - 50ng/µL were then sequenced by a commercial lab using the amplification primers. DNA sequences were assembled, edited and analyzed using gap4 and Pregap4 software's (part of Staden Package). The identity of contigs generated from sequence analysis was determined by comparison to a set of reference sequences listed on Lahey database (www.lahey.org) using CLC sequence viewer version 7 (Qiagen Inc., CA, USA).

2.3.5 Pulsed-field gel electrophoresis

261 isolates were selected for PFGE including all 99 3GC resistant isolates and 162 susceptible *E. coli*. PFGE was performed as described by PulseNet protocol using the CHEF-DRIII system (Bio-Rad Laboratories Ltd., ON, Canada) (CDC, 2016b). In brief, isolates from frozen stock culture (-80°C) were plated on blood agar and incubated overnight at 35°C. Colonies were suspended in 2ml of Gram-negative cell suspension broth and adjusted to 3 McFarland using a densitometer. 250µl of cell suspension were dispensed into a 1.5 ml micro-centrifuge tube and 11.4µl of proteinase-K added into it and then mixed with 227µl of melted (56°C) 1% SeaKem Gold Agarose (Lonza Rockland Inc., ME, USA) and quickly casted into the plug mold. Plugs were kept at room temperature until solidified. To begin the cell digestion, plugs were dispensed in 5ml Gram-negative cell lysis buffer containing 25µl of proteinase-K and incubated in water bath for ≥ 2 h at 56°C. After incubation, cell lysis buffer was decanted and

plugs were washed with distilled water for 15 minutes using a shaker with shaking at ~200rpm. The water was decanted, and the plugs were then washed 3 times with Gram-negative TE buffer for 15 mins each using a shaker with shaking at ~200rpm. Finally, plugs were stored in TE buffer at 4°C until used. 1/3 of each plug was subjected to restriction digest. For digestion, plugs were transferred into a 50ml polypropylene conical tubes containing 200µl of 1x equilibration buffer (10x buffer and BSA) and incubated for 10 minutes at 37°C. The equilibration buffer was then aspirated, and 200µl enzyme digestion solution (*Xba*I, 10x buffer and BSA) was then added; plugs were then incubated for ≥ 2 hours at 37°C. Following digestion, plugs were loaded in 1% agarose gel. The *Xba*I digested DNA fragments were separated by electrophoresis in 0.5X electrophoresis TBE buffer at 14°C for 20h on CHEF-DRIII electrophoresis system with an initial switch time of 2.16 s, a final switch time of 54.17 s, a 120° switch angle, and a gradient of 6.0 V/cm. *Salmonella enterica* serotype Branderup was used as reference ladder. Following electrophoresis, gels were stained with 600ml of distilled water containing 20µl of ethidium bromide for 20 mins and de-stained with 600ml of distilled water for 20 mins. DNA fragments were visualized with AlphaImager® HP (Fisher Scientific, ON, Canada) and photographed. The relatedness of all isolates was determined using Gel Compare II (Applied Maths Inc., TX, USA) based on Tenover criteria (Tenover et al., 1995). At least 3 isolates with ≥85% band similarity were defined as cluster (Tenover et al., 1995; Thorsteinsdottir et al., 2010). Cluster analysis was done using the Dice co-efficient with unweighted pair group method using arithmetic averages (UPGMA) and band tolerance 1.00%

2.3.6 Multi-locus sequence typing

Multi-locus sequence typing (MLST) was performed to define the PFGE clusters and identify the *E. coli* sequence type and clonal complexes. For MLST, 14 isolates were selected

from clusters forming units defined by PFGE and 10 isolates which did not form any clusters. Isolates were characterized by MLST using previously published scheme including seven housekeeping genes: *adk*, *fum*C, *gyr*, *icd*, *mdh*, *pur*A, *rec*A (Wirth et al., 2006). With the exception of *rec*A the amplification and sequencing were carried out using the primers listed in *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html) (**Table 2.2**) (Wirth et al., 2006). For *rec*A new primers were designed in-house and the reaction conditions were optimized. Allelic profiles and sequence types were determined via the online MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

2.3.7 Data analysis

Data obtained from the tests were organized using Microsoft excel and analyzed descriptively.

Table 2.2: PCR primers used to amplify seven housekeeping genes of *E. coli*

Primer Name	Sequence	Reference
adk	5’ -ATTCTGCTTGGCGCTCCGGG- 3’	(Wirth et al., 2006)
	5’ -CCGTCAACTTTCGCGTATTT- 3’	
fumC	5’ -GTACGCAGCGAAAAAGATTC 3’	(Wirth et al., 2006)
	5’ -TCACAGGTCGCCAGCGCTTC- 3’	
gyrB	5’ -TCGGCGACACGGATGACGGC- 3’	(Wirth et al., 2006)
	5’ -ATCAGGCCTTCACGCGCATC- 3’	
icd	5’ -ATGGAAAGTAAAGTAGTTGTTCCGGCACA- 3’	(Wirth et al., 2006)
	5’ -GGACGCAGCAGGATCTGTT- 3’	
mdh	5’ -ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG- 3’	(Wirth et al., 2006)
	5’ -TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT- 3’	
purA	5’ -CGCGCTGATGAAAGAGATGA- 3’	(Wirth et al., 2006)
	5’ -CATACGGTAAGCCACGCAGA- 3’	
recA	5’ -GTGCGTTTATCGATGCTGAA- 3’	This study
	5’ -TCTTTTACGCCCAGGTCAAC- 3’	

2.4 Results

2.4.1 PCR and DNA sequencing

Of the 99 3GC resistant isolates, 65 were originally cultivated using CHROMagar ESBL while 34 were grown on MacConkey agar. Of the 205 flocks sampled, broad-spectrum β-lactamase producing *E. coli* were isolated from 56 including 25 producing ESBLs, 30 producing AmpC type enzymes and 1 where both types were identified (**Table 2.3**). Phylogenetic analysis of CTX-M sequences revealed that predominant ESBL was CTX-M-1 in 54 isolates and the remaining 1 was closely related CTX-M group-1 genes (**Table 2.4**). CMY-2 was the only AmpC type enzyme identified (n=39). Four isolates contained both CTX-M-1 and CMY-2 genes. The majority of ESBL producers were detected from isolates grown on CHROMagar ESBL while the majority of AmpC producing isolates were recovered from MacConkey agar.

Table 2.3: Number of flocks positive for ESBL and AmpC β-lactamase producing *E. coli*

		AmpC Positive Flocks		Total
		(+)	(-)	
ESBL Positive Flocks	(+)	1	25	26 (12.7%)
	(-)	30	149	179 (87.3%)
	Total	31 (15.1%)	174 (84.9%)	205

Table 2.4: Frequency of β-lactamases genes in chickens from small flocks (<300 birds/year)

Samples isolated from	**No. of Samples resistant to 3GC**	**No. of samples positive for β-lactamases resistance genes**		
		CTXM type ESBL	CMY Type AmpC	Both ESBL and AmpC
CHROMagar ESBL	65 Φ	CTXM -1 (52; 80.0%) CTXM-1 like (1; 1.5%)	CMY- 2 (8; 12.3%)	CTXM-1 and CMY-2 (4; 6.2%)
MacConckey	34 ╫	CTXM -1 (2; 5.9%)	CMY- 2 (31; 91.1%)	-
Total	99	55 (55.6%)	39 (39.4%)	4 (4%)

Φ Two isolates from same sample were positive for ESBL and AmpC differently, so we included both two isolates in our study, ╫ Isolates from one sample out of 34 were not positive for either ESBL or AmpC.

2.4.2 Class I integrons

All 99 3GC resistant isolates were screened for class I integrons and amplicons were sequenced revealing 22 isolates possessing 1kb fragments. The *aad*A1 encoding the protein streptomycin 3"-adenylyltransferase which confers resistance to streptomycin and spectomycin was identified. Of the integron positive isolates, 11 also carried the CTX-M-1 gene and 11 isolates possessed CMY-2.

2.4.3 Pulsed-field gel electrophoresis

Of the 261 isolates characterized by PFGE, 226 yielded analyzable banding patterns including 80 of 99 β-lactamase producing isolates and 146 out of 162 non-resistant isolates. When the banding patterns from all 226 isolates were analyzed together, it was clear that the population structure was heterogeneous. A total of 9 clusters (groups of at least 3 isolates with ≥85% banding similarity), none of which contained more than 6 isolates were identified (**Figure 2.1**). A total of 23 β-lactamase producers were found within 7 clusters; only 3 of these clusters contained isolates from multiple flocks. Isolates with and without β-lactamase were found together in 1 cluster. One cluster comprised of five isolates harboring both class I integrons and the CTX-M-1 ESBL.

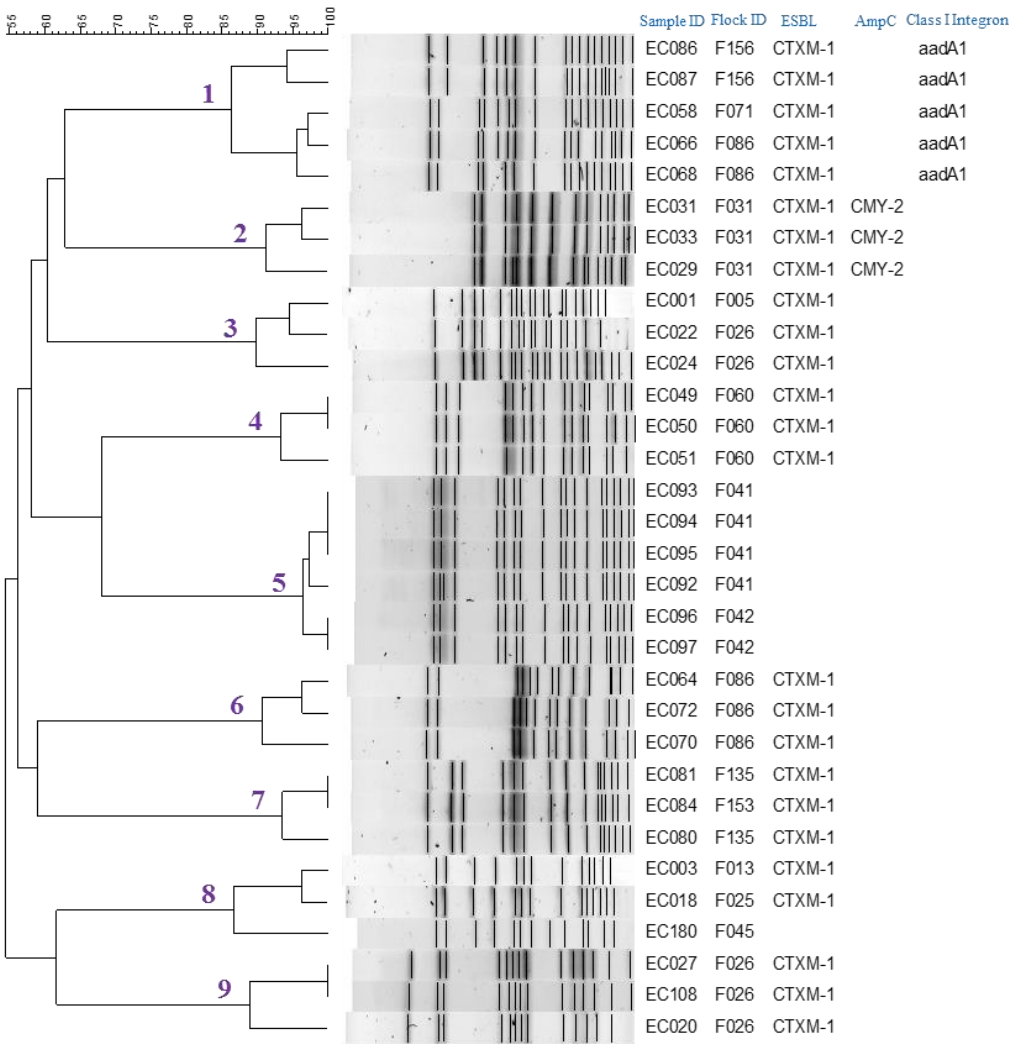

Figure 2.1: Relatedness of isolates as determined by PFGE. Only the cluster forming isolates (32 out of 226) are represented in this dendrogram. Cluster number is indicated by the violet coloured number. Restriction banding patterns (DNA fingerprints) are displayed in the middle. The presence of broad spectrum β-lactamases are indicated for each isolate by the ESBL and AmpC columns. Cluster analysis was done using GelCompare II, similarities were determined using the Dice co-efficient with unweighted pair group method using arithmetic averages (UPGMA) and band tolerance 1.00%.

2.4.4 MLST

To characterize the PFGE clusters, 24 isolates were selected. Of them 14 isolates from 9 clusters defined by PFGE and 10 non-clustering isolates were included. A sequence type was not assigned for one isolate, where *pur*A amplicons yielded poor quality sequence. Novel allelic combinations and therefore new sequence types were identified in 6 isolates (**Table 2.5**). MLST revealed that all isolates clustering together by PFGE were the same sequence type except for cluster number 3 which were closely related two locus variants. There were no predominant STs particularly associated with CTX-M-1 productions in Canadian small chickens. Non-clustering isolates all had unique sequence types. Four different clonal complexes were identified, while ST10 clonal complex was observed in both cluster and non-cluster forming isolates.

Table 2.5: Distribution of *E. coli* sequence types in chickens from small scale farms

Cluster Number	Sample ID	*adk-fumC-gyr-icd-mdh-purA-recA*	Sequence Type	Clonal complex	β-lactamase gene
1	EC-008	6-31-5-28-1-1-2	ST57	ST350 complex	CTX-M-1
1	EC-087	6-31-5-28-1-1-2	ST57	ST350 complex	CTX-M-1
2	EC-033	6-4-4-16-24-8-14	ST58	ST155 complex	CTX-M-1 & CMY-2
3	EC-001	87-7-231-140-1-187-14	***ST2792 -2**		CTX-M-1
3	EC-024	87-53-231-140-1-187-2	ST2792		CTX-M-1
4	EC-050	6-11-4-8-8-8-6	ST3270		CTX-M-1
5	EC-093	6-4-159-44-112-1-17	ST1011		ND
5	EC-097	6-4-159-44-112-1-17	ST1011		ND
6	EC-064	6-95-4-18-11-7-14	ST1304		CTX-M-1
7	EC-080	6-65-344-1-11-13-6	ST3580		CTX-M-1
8	EC-003	6-4-14-16-24-8-14	ST155	ST155 complex	CTX-M-1
8	EC-180	6-4-14-16-24-8-14	ST155	ST155 complex	ND
9	EC-020	6-11-4-8-8-8-2	ST48	ST10 complex	CTX-M-1
9	EC-108	6-11-4-8-8-8-2	ST48	ST10 complex	CTX-M-1
NC	EC-038	6-11-4-8-8-**N**-2	***ST48 - 1**		CTX-M-1
NC	EC-055	6-65-32-26-9-8-2	ST297		CTX-M-1
NC	EC-068	6-31-5-28-1-1-2	ST57	ST350 complex	CTX-M-1
NC	EC-107	6-4-15-1-22-1-7	***ST5617 -1**		CMY-2
NC	EC-120	10-53-4-8-12-8-2	***ST746 -1**		CMY-2
NC	EC-165	6-4-5-26-20-8-14	ST40	ST40 Complex	ND
NC	EC-192	6-11-4-8-8-8-2	ST48	ST10 Complex	ND
NC	EC-199	111-7-4-8-12-8-2	***ST813 -1**		ND
NC	EC-203	6-11-15-1-22-8-7	***ST366 -1**		ND
NC	EC-209	10-4-4-8-8-8-2	ST178	ST10 Complex	ND

NC = Non-cluster, ND = Not detected; N = poor quality sequence which was not assigned an allelic number; *indicates a novel allelic combination, the most similar sequence type and the number of loci differing between them are indicated.

2.5 Discussion

In Canada, chicken originating from small-scale farms which pass through provincial regulated abattoirs are not included in the current national antimicrobial surveillance program (Government of Canada, 2015a). Moreover, there were no available data for antimicrobial resistance for these backyard chickens. To address this gap, we conducted this study to identify broad-spectrum β-lactamase producing *E. coli* from this understudied bird population.

We found that among *E. coli* resistant to 3GC isolated from chickens raised in small flocks in southern Ontario, ESBL producing *E. coli* were isolated from 12.7% flocks tested. In contrast, researchers in India were able to detect ESBL producing isolates from 29.4% of commercial flocks but failed to identify any ESBLs among birds raised in *E. coli* from birds raised in free-range (Samanta et al., 2015). Low levels of resistance were previously reported in backyard poultry comparing with intensively farmed poultry in Australia (Obeng et al., 2012). Both these studies reported very limited or occasional use of β-lactam drugs in those backyard chicken population which has been previously reported to be associated with the presence of resistance to these drugs (Agersø et al., 2012; Cohen Stuart et al., 2012; Lowrance et al., 2007; Obeng et al., 2012; Samanta et al., 2015). In Canada, β-lactam drugs including aminopenicillins, ceftiofur has been reported as commonly used drugs to treat *E. coli* infection in chickens (Agunos et al., 2012). This might be one of the reasons for the seemingly high frequency of ESBL producing *E. coli* in Canadian backyard chickens. However, there is no data available for antimicrobial use in chickens coming from small-scale farms to support this hypothesis.

ESBLs producing *E. coli* were observed in higher percentage from the isolates came through CHROMagar ESBL (selective media), while AmpC producers were more common in isolates grown on MacConkey agar. As CHROMagar ESBL particularly designed to screen

ESBLs by inhibiting the growth of AmpC producers (CHROMagar, 2012), finding lower number of AmpC producers on CHROMagar ESBL was therefore an expected outcome. Previous studies focusing on ESBL and AmpC producers, found that ESBL producing isolates were more common than AmpC producers in broiler flocks (Reich et al., 2013).

CTX-M-1 and CMY-2 were reported as the most frequently detected genes in 3GC resistant *E. coli* strains from chickens raised in small-scale flocks. Our findings are consistent with those of a French study where CTX-M and CMY-2 were the predominant broad-spectrum β-lactamases in 3GC resistant *E. coli* in poultry (Baron et al., 2014). Other studies in Europe reported a high frequency of CTX-M-1 among *E. coli* from chickens (Belmar Campos et al., 2014; Girlich et al., 2007; Randall et al., 2011). Though CTX-M-1 has been frequently encountered in animals globally, however, few studies found this gene to a lesser extent from human *E. coli* isolates (Belmar Campos et al., 2014; Blanc et al., 2014; Madec et al., 2015; Valentin et al., 2014). CTX-M-15 has been reported to be the most commonly encountered human ESBL type around the world (Belmar Campos et al., 2014; Geser et al., 2012b; Reuland et al., 2013). In Canada, CTX-M-15 was also identified as the most common ESBL among human clinical isolates (Peirano et al., 2010; Zhanel et al., 2010), while our study identified a different predominant gene in *E. coli* from poultry. As humans come into close contact with backyard chickens, there is a risk of zoonotic transmission of pathogenic bacteria or other organisms harboring mobile resistance genes (Pohjola et al., 2016). However, as we found that the predominant ESBLs identified from these birds are different from those most commonly encountered in people, this risk may be lower than previously assumed.

To better understand the relatedness of the resistant isolates in our current study, PFGE was performed and 226/261 isolates yielded analyzable banding patterns. Failure to in obtaining

analyzable data is consistent with previous studies which found that some strains are non-typeable by PFGE (Bens et al., 2006; CDC, 2016b). Heterogenous fingerprint patterns were found for resistant and susceptible isolates, while few isolates were found to be closely related which were together defined as cluster. Of the 9 isolate clusters, 4 contained isolates from the same flock, while 5 contained isolates from multiple flocks. Close contact between birds within a flocks perhaps explains these findings. The presence of the same β-lactamase gene was identified in unrelated isolates, suggesting that the dissemination of this resistance in *E. coli* colonizing chickens was associated with the spread of plasmids rather than particular *E. coli* clones. These findings were consistent with previous studies which found that dissemination of CTX-M genes were associated with horizontal transfer of plasmids rather than clonal spread (Girlich et al., 2007; Liebana et al., 2006), which strengthen our current statement regarding spread of CTX-M type ESBL via horizontal gene transfer in Canadian chicken isolates.

MLST revealed that the majority of the isolates which clustered together by PFGE were the same sequence types. Our current findings are in consistence with other studies where they found ST10, ST57, ST58, ST155 as predominant STs from chicken *E. coli* isolates associated with ESBL production (Ben Sallem et al., 2014; Overdevest et al., 2011). Four different clonal complexes, ST155, ST10, ST350 and ST40 were identified while ST10 clonal complex was observed both in cluster forming and non-cluster forming units. Other studies in Canada reported ST10 clonal complexes from human clinical infections as well as chicken and franklin gulls (Bergeron et al., 2012; Bonnedahl et al., 2015). However, ST131 which is a frequently reported *E. coli* from community onset UTIs globally including Canada, and is often associated with CTX-M-15 production (Graham et al., 2016; Peirano et al., 2010; Pitout, 2012a). Interestingly,

none of our isolates were identified as ST131 which do not support our hypothesis of findings human resistant pandemic clones in Canadian chicken.

The risk to Canadian public health associated with this resistance infection coming from poultry has not yet been adequately investigated. Our study identified CTX-M-1 as predominant ESBL type in Chicken which is different than human population. Fingerprinting results showed isolates in this current study were heterogeneous. Overall our findings suggest that isolates from small flocks pose relatively a low public health risk, although further studies are required to determine any link on ESBL producing *E. coli* from Canadian human and chicken population. The dissemination of a single ESBL (CTXM-1) in chickens from different small-scale flocks could be due to horizontal transmission of resistant genes between birds possibly occurring at the hatchery level. The current study also suggests the importance of using selective media in AMR studies to identify an important type of resistance gene such as CTX-M-1 while these genes might be undetected if using only non-selective media such as MacConkey.

2.6 Transition Statement

In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) monitors the antimicrobial susceptibility of *E. coli* from commercial broiler chickens slaughtered at federally inspected abattoirs. However, CIPARS does not routinely include any description of resistance mechanisms on these isolates which is very important in order to deeper understanding of the epidemiology of antimicrobial resistance. Additionally, CIPARS does not use any selective media to screen the β-lactamases producing isolates which may lead to an underestimation of the prevalence of these enzymes. In our first study, selective media was used to identify 3rd generation cephalosporin resistant isolates from small-scale flocks which were then screened for β-lactamase genes by PCR. This study on small scales chicken flocks highlighted a potential gap in identifying β-lactamase producing *E. coli* from chickens raised in large commercial flocks. In the next chapter, we determined broad-spectrum β-lactamase producing genes from large commercial chickens using selective media to address these gaps.

3 Broad spectrum β-lactamase producing *Escherichia coli* isolated from large-scale commercial chickens in Ontario

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Conceived and designed the experiments: KKG, JER, SG, RJR. Sample preparation: NJ, AA. Performed the experiments: KKG. Analyzed the data: KKG, JER. Wrote the paper: KKG.

3.1 Abstract

Broad spectrum β-lactamase producing *E. coli* are emerging in humans and food producing animals throughout the world. The objective of this study was to identify ESBL and AmpC producing *E. coli* from commercial broiler chickens raised in Ontario, Canada and to compare the use of selective media with the standard CIPARS protocol. Ceacal contents from birds passing through federally inspected abattoirs collected in 2013 and 2014 by CIPARS were included. These archived ceacal samples were sent to the University of Saskatchewan to selectively culture *E. coli* resistant to the 3GC and identify the broad-spectrum β-lactamases in these isolates. A total of 134 isolates were grown on media selective for ESBL producing organisms were recovered from 52 of 463 samples (11.4%). Antimicrobial MICs were determined for all isolates using the Sensititre® system. A high frequency of resistance towards β-lactam drugs; ampicillin (100%), ceftriaxone (92.5%) and ceftiofur (91.7%) was identified. Multidrug resistance (resistant to ≥ 3 classes) was observed in 94.8% of isolates. ESBL producing isolates were recovered from 35/463 (7.6%) of samples while 12/463 samples were positive for AmpC. All ESBL genes detected were identified as CTX-M-1 while 61.3% of AmpC genes were CMY-2. In conclusion, our study suggests that using selective media is helpful for identifying CTX-M-1 mediated 3GC resistance, which might not be detected using non-selective media.

3.2 Introduction

Antimicrobial resistance is an emerging problem throughout the world including Canada. In addition to the prospect of untreatable infections, antimicrobial resistance results in higher healthcare costs due to longer hospital stays, the requirement for additional diagnostics and more expensive drugs (WHO, 2016). β-lactam group drugs are commonly used to treat *E. coli* infection in both humans and animals (Briñas et al., 2002). However, the emergence of extended-spectrum β-lactamase (ESBL) producing *E. coli* is a concern in both human and veterinary medicine as it confers resistance to most of the β-lactam drugs including 3rd and 4th generation cephalosporin (Blaak et al., 2015). AmpC type β-lactamases are also problematic conferring resistance to many of the same drugs (Rawat and Nair, 2010).

TEM (except TEM 1, 2 and 13), SHV (except SHV 1) and CTX-M are common examples of ESBLs, the CTXM type enzymes have been dominant worldwide since the early 2000's (Rubin and Pitout, 2014). CMY, FOX, ACT, MOX, ACC, DHA are examples of AmpC type β-lactamase (Rubin and Pitout, 2014). In poultry and other animals CTX-M-1 was reported as most encountered ESBL type in *E. coli* in Europe (Blaak et al., 2014; Dierikx et al., 2013; Overdevest et al., 2011; Randall et al., 2017). In contrast, the majority of human isolates have been found carry CTX-M-15 (Valentin et al., 2014). CTX-M-15 has also been identified in the USA and Canada as most prevalent resistant gene in humans and associated with the pandemic *E. coli* sequence type 131 (ST131) (Denisuik et al., 2013; Overdevest et al., 2011). Among AmpC type β-lactamase in *E. coli* from hospitalized Canadian patients, CMY-2 was the prevalent variant (Denisuik et al., 2013). Another study from Norway found CMY-2 producing extended-spectrum cephalosporin-resistant *E. coli* from retail chicken which were closely related with human clinical isolates carrying the same gene suggesting that transmission between poultry and

humans is occurring (Berg et al., 2017). Similarly, ESBL producing *E. coli* from Dutch retail chicken meat were found to be indistinguishable to human clinical isolates (Overdevest et al., 2011). These findings suggest food producing animals particularly chicken meat might be a source of resistance genes for humans.

The Canadian chicken industry is growing and contributes $6.8 billion to Canada's Gross Domestic Product (GDP) (Chicken Farmers of Canada, 2016b). In Canada, per capital consumption of chicken meat is 31.86 kilos while beef and pork consumptions are 24.40 and 22.63 kilos respectively (Government of Canada, 2016b). Chicken is an increasingly large portion of the diet and is therefore particularly important as a source of resistance in Canada. Ontario is the largest chicken producer in Canada with 1,155 chicken farmers and 42 processors, contributing $2.2 billion to Canada's GDP annually (Chicken Farmers of Canada, 2016b).

Antimicrobial resistance surveillance in *E. coli* and other food borne bacteria is important for tracking the emergence of resistance and assessing the impact of resistance control strategies (Government of Canada, 2015b). The Canadian Integrated Program for Antimicrobial Surveillance (CIPARS) is the national resistance surveillance program which monitors antimicrobial use and resistance in enteric bacteria from food animals on farm and at slaughter, retail meat and human clinical isolates at federal level. Two types of slaughter facilities (federal and provincial) exist in Canada (Government of Canada, 2015b). More than 90% food producing animals in Canada are processed in these federally inspected abattoirs which included in CIPARS sampling (Government of Canada, 2015a).

CIPARS only uses non-selective media to screen resistant isolates and molecular characterization of resistant isolates is also a limitation of their program. We conducted this study to determine whether the inclusion of selective media will improve our ability to detect *E.*

coli producing broad-spectrum β-lactamases from large commercial chickens which pass through federally inspected abattoirs.

3.3 Materials and Methods

3.3.1 Study design

This is a retrospective comparison where samples were re-analyzed using a different methodological strategy to compare approaches. Further, this study was performed to determine if different genes are responsible for 3GC resistance among *E. coli* isolated from commercial broiler chickens. Archived cecal samples collected by CIPARS from chickens in Ontario in 2013 and 2014 were included. Broad spectrum β-lactam resistant *E. coli* were isolated and identified using selective culture media. Antimicrobial MICs were determined by broth microdilution. Isolated were screened for β-lactamase genes by PCR and DNA sequencing.

3.3.2 Sample collection and processing by CIPARS

Chicken caecal contents included in this study were collected by CIPARS from federally inspected abattoirs in Ontario in 2013 and 2014. In brief, caecal content from chickens were collected to reduce the chances of cross contamination and to better reflect antimicrobial resistance in bacteria that originated on the farm (Government of Canada, 2015a). Samples were streaked out onto MacConkey agar and *E. coli* positive isolates were identified. Antimicrobial MICs were determined using broth microdilution. Following routine processing, CIPARS froze back cecal contents for future studies (Government of Canada, 2015a).

3.3.3 Culture and isolation of *E. coli* using selective and non-selective media

Frozen caecal samples were shipped from CIPARS on dry ice to the University of Saskatchewan. On arrival, samples were streaked on MacConkey agar plate to ensure that viable *E. coli* were present. After incubation at 37°C for overnight, isolates from pink colonies were identified and biochemically tested using the citrate, urease and indole tests. Any isolates with citrate negative, urease negative, and indole positive were identified as *E. coli* confirming the integrity of the sample.

All samples were also streaked out onto CHROMagar ESBL, a selective media to detect ESBL producing *E. coli*. Dark pink colonies were considered to be suspected ESBL producers, up to 3 colonies were picked and sub-cultured onto 5% sheep agar plates. Colonies from each blood agar plates were then identified biochemically and *E. coli* was stored for further analysis.

3.3.4 Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was performed by broth microdilution using the Sensititre system (Trek Diagnostics, Cleveland, OH). The NARMS plate (CMV2AGNF format) which includes 15 drugs representing 7 classes was used in this study according to the manufacturer's instructions (**Table 3.1**). In brief, isolates were sub-cultured on blood agar plate and incubated overnight at 37°C. 3-4 colonies were then suspended in 5ml demineralized water to a density of 0.5 McFarland to prepare the inoculum. 30 µl of bacterial suspension was added to 11ml cation-adjusted Muller Hinton broth (Trek Diagnostics, OH, USA). 50 µl of inoculum was dispensed into the 96 well CMV2AGNF plate using the Sensititre auto-inoculator. Plates were sealed with adhesive film and incubated at 35°C for 18-22 hours. Antimicrobial MICs were

interpreted (classified as susceptible or resistant) according to CLSI guidelines (**Table 3.1**). For quality control, *S. aureus* ATCC 29213 and *E. coli* ATCC 25422 were used.

Table 3.1: List of drugs with resistance breakpoints used in antibiotic susceptibility testing panel

Drug Class	Drug	Tested Range	Breakpoint
β-lactams	Cefoxitin	0.5 - 32	≥32
	Ceftriaxone	0.25 - 64	≥4
	Amoxicillin-Clavulanic acid	1/0.5 - 32/16	≥32/16
	Ceftiofur	0.12 - 8	≥8
	Ampicillin	1 - 32	≥32
Phenicol	Chloramphenicol	2 - 32	≥32
Tetracyclines	Tetracycline	4 - 32	≥16
Sulfonamides	Sulfisoxazole	16 - 256	≥512
	Sulfisoxazole-Trimethorprim	0.12/2.38 - 4/76	≥4
Quinolones	Ciprofloxacin	0.015 - 4	≥4
	Nalidixic Acid	0.5 - 32	≥32
Macrolides	Azithromycin	0.12 - 16	≥32
Aminoglycosides	Gentamicin	0.25 - 16	≥16
	Kanamycin	8 - 64	≥64
	Streptomycin	32 - 64	≥64

3.3.5 Bacterial DNA extraction and polymerase chain reaction of targeted gene sequences

Crude DNA extracts (boil preps) of cultures were made in sterile distilled water. In brief, bacteria were sub-cultured on blood agar plate overnight at 37°C. In a sterile 1.5ml micro-centrifuge tube, 200uL of ultrapure water was added and several isolated colonies were suspended in it. Tubes were then vortexed and boiled in a heat block for 10 minutes. After boiling, tubes were cooled at room temperature for 2 minutes, and centrifuged for 1 minute at 13000rpm. The supernatant was then transferred to a sterile 1.5 ml micro-centrifuge tube and stored at -20°C until used.

Previously published primers of CTXM-U, CTXMG-1 and CMY-2 gene were used to detect ESBL and AmpC genes (**Table 3.2**). PCR reactions were carried out in 25µL volumes including 24µL of mastermix and 1µL of boil prep DNA template was added. In each set of reactions, positive and negative controls were included. Thermycycler conditions were as follows: initial denaturation at 94°C for 6 mins followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C (CTX-M-U, CTX-M-G1)/68°C (CMY-2) for 1 min, elongation at 72°C for 1 min and final extension at 72°C for 10 mins. Amplicons were resolved by electrophoresis using 1% agarose gel at 110 volts for 30 minutes. 3µL of DNA gene Ladder was used to the first well. Gels were then visualized under UV light using the AlphaImager® HP (Fisher Scientific, Toronto, ON).

Table 3.2: PCR primers used to detect ESBL and AmpC β-lactamase genes in *E. coli*

Primer Name	Primer Sequence	References
CTXM-U	5’ -ATGTGCAGYACCAGTAARGTKATGGC- 3’	(Munday et al., 2004)
	5’ -TGGGTRAARTARGTSACCAGAAYCAGCGG- 3’	
CTXM-G1	5’ -GTTGTTAATTCGTCTCTTCC- 3’	(Pitout et al., 2004)
	5’ -AGTTTCCCCATTCCGTTTC- 3’	
CMY-2	5’ -ATGATGAAAAAATCGTTATGCTGC- 3’	(Kruger et al., 2004)
	5’ -GCTTTTCAAGAATGCGCCAGG- 3’	(Hasman et al., 2005)

3.3.6 PCR amplicon purification and nucleotide sequencing

PCR products were purified using EZ-10 Spin Column PCR purification kit (Bio Basic Canada Inc., ON, Canada) and DNA was quantified by spectrophotometrically (NanoDrop™ 1000). Purified DNA with ~1.8 ratio of absorbance at 260 nm and 280 nm and 2.0 - 2.2 of 260/230 values were considered to have sufficiently high quality for sequencing. DNA with a concentration of 10 - 50ng/µL was then sequenced by a commercial lab using the amplification primers. DNA sequences were assembled, edited and analyzed using gap4 and Pregap4 software's (part of Staden Package). The identity of contigs generated from sequence analysis was determined by comparison to a set of reference sequences listed on Lahey database (www.lahey.org) using CLC sequence viewer.

3.3.7 Data analysis

All the results were recorded in Microsoft Excel, and analyzed descriptively.

3.4 Results

3.4.1 Recovery of *E. coli* using selective and non-selective media

Of the 466 samples tested, *E. coli* was recovered from 463 samples. Lactose fermenting colonies from the remaining 3 samples were observed but were biochemically identified as other Enterobacteriaceae species. The recovery rate of the samples was 99.4%. Out of 463 samples, 52 were found positive on CHROMagar ESBL. Up to 3 isolates were isolated from each sample positive on CHROMagar, a total of 134 isolates were identified and frozen at -80°C (**Table 3.3**).

Table 3.3: Number of *E. coli* isolates in chickens from large-scale flocks using selective media (CHROMagar ESBL)

Total sample number	466
Samples from which *E. coli* was recovered	463
Samples from which *E. coli* was recovered on CHROMagar ESBL	52
Number of isolates recovered from CHROMagar ESBL	134*

*Up to 3 isolates were isolated from each CHROMagar positive sample

3.4.2 Antibiotic resistant profile of broad-spectrum β-lactamase producing *E. coli*

3.4.2.1 MIC distribution based on drug classes

The antimicrobial minimum inhibitory concentrations of the 134 isolates recovered from CHROMagar were determined, and interpreted based on CLSI guidelines. The MIC distributions of isolates to drugs categorized by class are presented in **Table 3.4**. Overall, no pan-susceptible isolates were found, 100% of isolates were resistant to ampicillin while ceftriaxone and ceftiofur resistance was observed in 92.5% and 91.7% of isolates respectively. The frequency of resistance to cefoxitin and amoxicillin-clavulanic acid was similar including 28.4 and 29.1% of isolates respectively. Resistance to sulfamethoxazole-trimethoprim was observed in 23.9% of isolates, while 94% of isolates were resistant to potentiated sulfisoxazole. Resistance to tetracycline was similarly high observed in 92.5% of isolates. Within a sample, the susceptibility of the three isolates recovered was generally homogenous.

Table 3.4: Minimum inhibitory concentration distribution of *Escherichia coli* (n=134) from chickens raised in large-scale farms

Drug Class	Name	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	%R Isolates (n=134)	%R Samples (n=52)
β-lactam	Cefoxitin								4	51	34	7	38				38 (28.4)	16 (30.8)
	Ceftriaxone						2	3	5		1	12	34	77			124 (92.5)	48 (92.3)
	Amox Clav									6	77	12	39				39 (29.1)	16 (17.4)
	Ceftiofur							1	7	3	123						123 (91.7)	48 (92.3)
	Ampicillin												134				134 (100)	52 (100)
Sulfonamides	Sulfisoxazole											8				126	126 (94.0)	49 (94.2)
	SXT				52	46	4			32							32 (23.9)	13 (25.0)
Quinolones	Ciprofloxacin	114	17							3							3 (2.2)	1 (1.9)
	Nalidixic Acid							7	91	32	1		3				3 (2.2)	1 (1.9)
Aminoglycosides	Gentamycin					3	47	31	5	1	1	46					46 (34.3)	19 (36.5)
	Kanamycin										107	17	2	8			10 (7.5)	5 (9.6)
	Streptomycin												86	48			48 (35.8)	24 (46.2)
Tetracyclines	Tetracycline									10		1	123				124 (92.5)	49 (94.2)
Phenicol	Chloramphenicol								1	19	93	6	15				15 (11.2)	6 (11.5)

Antimicrobial concentrations for each drug tested are indicated by cells falling between shaded boxes. The red boxes indicate concentrations corresponding to resistance. The number of isolates inhibited at each concentration is noted in each cell. %R Isolates indicates the number of resistant isolates while %R Samples indicates the number of animals from which an isolate resistant to each drug was isolated.

3.4.2.2 MIC distribution based on importance of drugs to human health

Drugs were also categorized by PHAC based on their importance to human health and the MIC distribution was observed based on this in **Table 3.5**. Resistance to a number of drugs classified as "category I - very high importance to human health" was found. Ceftiofur and ceftriaxone resistance was identified among 91.7 and 92.5% of isolates respectively. Further, isolates were susceptible to drugs listed on "category II - high importance to human health" except ampicillin.

Table 3.5: Minimum inhibitory concentration distribution of *Escherichia coli* (n=134) based on PHAC Drug Category

Importance to Human Health*	Name	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	%R Isolates (n=134)	%R Samples (n=52)
I – Very high importance	Ceftiofur							1	7	3	123						123 (91.7)	48 (92.3)
	Ceftriaxone						2	3	5		1	12	34	77			124 (92.5)	48 (92.3)
	Amox Clav									6	77	12	39				39 (29.1)	16 (17.4)
	Ciprofloxacin	114	17							3							3 (2.2)	1 (1.9)
II – High importance	Ampicillin												134				134 (100)	52 (100)
	Cefoxitin								4	51	34	7	38				38 (28.4)	16 (30.8)
	Naladixic Acid							7	91	32	1		3				3 (2.2)	1 (1.9)
	Gentamicin					3	47	31	5	1	1	46					46 (34.3)	19 (36.5)
	Kanamycin										107	17	2	8			10 (7.5)	5 (9.6)
	Streptomycin												86	48			48 (35.8)	24 (4.6)
	SXT				52	46	4			32							32 (23.9)	13 (25.0)
III – Medium importance	Chloramphenicol								1	19	93	6	15				124 (92.5)	49 (9.4)
	Tetracycline									10		1	123				15 (11.2)	6 (11.5)
	Sulfisoxazole											8				126	126 (94.0)	49 (94.2)

* Health Canada's Veterinary Drugs Directorate categorized these drugs based on their importance to human health (Government of Canada, n.d.). Antimicrobial concentrations for each drug tested are indicated by cells falling between shaded boxes. The red boxes indicate concentrations corresponding to resistance. The number of isolates inhibited at each concentration is noted in each cell. %R Isolates indicates the number of resistant isolates while %R Samples indicates the number of animals from which an isolate resistant to each drug was isolated.

3.4.2.3 Percentage of isolates and samples resistance to different antimicrobial classes

No isolates were fully susceptible to all drugs were found. Multidrug resistance, defined as resistance to three or more drug classes (Tadesse et al., 2012), was observed in 127 (94.8%) of isolates from 50 (96.2%) of samples. Resistance to 5 antimicrobial classes was identified in 14/134 (10.4%) of isolates from 5 (9.6%) of samples (**Table 3.6**). No pan-resistant isolates were identified.

Table 3.6: Percentage of isolates and samples resistance to different antimicrobial classes

Resistance to Antimicrobial Classes*	Isolates (n=134) N (%)	Samples (n=52) N (%)
Fully sensitive	0	0
Resistant to 1 Antimicrobial Class	4 (2.9)	1 (1.9)
Resistant to 2 Antimicrobial Classes	3 (2.2)	1 (1.9)
Resistant to 3 Antimicrobial Classes	62 (46.3)	21 (40.4)
Resistant to 4 Antimicrobial Classes	51 (38.1)	24 (46.2)
Resistant to 5 Antimicrobial Classes	14 (10.4)	5 (9.6)
Resistant to 6 Antimicrobial Classes	0	0

* Drug Classes: β-lactam, sulfonamides, quinolones, aminoglycosides, tetracycline, phenicol

3.4.3 Broad-spectrum β-lactamase producing genes by PCR and DNA sequencing

All 134 isolates were screened for ESBL and AmpC β-lactamases, 89 (66.4%) isolates from 35 (7.6%) of samples were found to carry ESBL encoding genes while 31 (23.1%) were positive for AmpC from 12 (2.7%) samples. DNA sequencing results revealed that all 89 ESBL producing isolates possessed CTX-M-1. 31 isolates were found to harbor CMY type enzymes which were determined to be CMY-2 (n=20) and CMY-61 (n=12). Co-location of ESBL and AmpC β-lactamases was observed in 3 isolates (2.2%) from 2 (0.4%) samples (**Table 3.7**).

Table 3.7: Number of ESBL and AmpC β-lactamase producing *E. coli* in chickens from large commercial broilers farms in Ontario, Canada

Classes of β-lactamase genes tested	Number of samples positive for β-lactamases genes* (n=463)	Number of isolates positive for β-lactamases genes (n = 134)	β-lactamase alleles
ESBL	35 (7.6%)	89 (66.4%)	CTXM -1 (89; 100%)
AmpC	12 (2.6%)	31 (23.1%)	CMY-2 (19; 61.3%) CMY -61 (12; 38.7%)
Both ESBL and AmpC	2 (0.4%)	3 (2.2%)	CTXM - 1 & CMY-61 (2; 66.7%) CTXM-1 & CMY-2 (1; 33.3%)

* Unique isolates from two samples (sample ID: FB393 & FB261) were positive for ESBL and AmpC individually, those two isolates are therefore represented in both the ESBL and AmpC cells of this table.

3.5 Discussion

Samples collected by CIPARS in 2013 and 2014 from chicken abattoirs in Ontario were included in this study. Culture on MacConkey agar resulted in a 99.4% recovery rate indicating that sample integrity was intact. This *E. coli* recovery rate was similar to what CIPARS found in the year 2013 (99%; 171/172) indicating that sample viability was not affected by long term storage. Other studies also found higher *E. coli* recovery rate from frozen samples (Green et al., 2007; Lautenbach et al., 2008), which suggests that frozen samples could be used without loss of integrity for a longer period of time.

In our current study, high resistance to ceftiofur and ceftriaxone and low resistance to cefoxitin and amoxicillin-clavulanic were found. This was expected as selective media (CHROMagar ESBL) was used in this study which can detect ESBL producing *E. coli* while suppressing the growth of AmpC producing *E. coli* (CHROMagar, 2012). The inhibitory effect of CHROMagar ESBL on AmpC type enzymes could result in selecting against isolates resistant to cefoxitin and amoxicillin-clavulanic. In their abattoir surveillance in 2013, CIPARS reported that 20.7% of isolates were resistant to these drugs (Government of Canada, 2015b). The current study suggests that using selective media to identify broad spectrum β-lactamase producing *E. coli* may be beneficial for detecting important resistance determinants not currently captured under current CIPARS surveillance activities. In the USA, a trend of increasing MDR in *E. coli* was observed from the 1950s when 7.2% of isolates were MDR to the 2000s when 63.6% of isolates were MDR (Tadesse et al., 2012). This is indicating that MDR is emerging among poultry isolates. In current study, majority of the isolates (94.8%) grown on CHROMagar ESBL showed MDR which suggests that selective media could aid in tracking MDR organisms. Using selective media, our study found ESBL producers 7.6% (35/463) of the *E. coli* positive samples.

Our current findings are in contrast with few European studies where a higher prevalence (>60%) of ESBL producing Enterobacteriaceae from chicken retail meat were identified (Doi et al., 2010; Overdevest et al., 2011). Geographical location, farm density and management practices might be the reasons behind lower ESBL resistance in Canada.

The dominance of CTM-X-1 in our study concurs with other reports where CTX-M-1 was found as the most prevalent ESBL type in chicken and other food producing animals (Agersø et al., 2012; Belmar Campos et al., 2014; Horton et al., 2011; Kola et al., 2012; Valentin et al., 2014). Additionally, our findings are in consistent with other studies where they found CMY-2 producing *E. coli* as predominant AmpC type in Chicken (Börjesson et al., 2013; Botelho et al., 2015). The extensive use of broad-spectrum cephalosporins was postulated as a reason for with the spread of CTX-M type ESBL (Hsu et al., 2010; Zhanel et al., 2010). A significant relationship between use of ceftiofur in chicken and incidence of ceftiofur resistant in humans and retail chickens was identified in Quebec indicating the link between drug animals and people (Dutil et al., 2010). However, CTX-M-15 *E. coli* is the most frequently encountered ESBL type in people worldwide including Canada (Leistner et al., 2014; Peirano et al., 2012, 2010; Valentin et al., 2014; Voets et al., 2012; Zhanel et al., 2010), while this study encountered a different ESBL type (CTX-M-1) in *E. coli* from Canadian large commercial broiler chickens. Different ESBL type in Canadian chickens comparing with human AMR data suggests that large commercial broiler flocks may not pose a high public health risk. However, more studies are required to evaluate the transmissibility of resistant gene baring mobile genetic elements between *E. coli* from commercial broiler chickens in Canada and humans.

Our study demonstrates that surveillance programs may find it useful to include selective media to identify organisms resistant to drug classes of particular interest, including ESBL

producing *E. coli*. One study found using selective media could help to determine ESBL producing *E. coli* (Randall et al., 2017). A variety of chromogenic media including CHROMagar ESBL, Brillance ESBL can be used as selective media for the detection of ESBL producing *E. coli*. The inclusion of ceftazidime and cefotaxime can help in detecting CTX-M type ESBLs whereas cefpodoxime alone can be used to detect other TEM and SHV type ESBLs (European Centre for Disease Prevention and Control, 2014).

In this study CTX-M-1 was found to be the predominant ESBL type in *E. coli* from chickens raised large flocks in Ontario; consistent with our previous investigation where the same gene was identified in isolates from small flocks. Chickens from large and small scales flocks are different in terms of bird population, management system, drug use, veterinary care. However, finding same resistant determinants suggests that resistant genes encoding on mobile genetic elements could be disseminated via horizontal transfer within bird's population.

4 General Discussion and Conclusion

4.1 General discussion

Antimicrobial resistance in Gram-negative bacteria is increasing throughout the world (Kuenzli, 2016; Laxminarayan et al., 2013; Paterson, 2006). Food producing animals including chickens are important zoonotic reservoirs of drug resistance determinants in the community. In Canada, the majority of the birds are slaughtered in federally inspected abattoirs while a small portion originating from small scale flocks pass through provincially inspected facilities (Government of Canada, 2015a). Although birds passing through federal inspected abattoirs are routinely included in resistance surveillance programs, no data are available about the antimicrobial resistance genes responsible for these phenotypes. (Government of Canada, 2015a). Another gap is the failure to include *E. coli* from chickens reared in small flocks in current surveillance programs. Several factors which may be associated with a different level of risk of resistance in small flocks include poorer biosecurity, high disease burden and less veterinary consultation. Furthermore, the relatively close contact with these chickens may be associated with unique risks. Finally, the increasing volume of chicken consumed highlights the increasing opportunities for the zoonotic transmission of poultry *E. coli* (Bergeron et al., 2012). In these present investigations, these gaps were addressed by studying *E. coli* from Ontario chickens.

We performed our first study on characterizing broad spectrum β-lactamase producing *E. coli* isolated from chickens raised in small scale flocks. Our study identified ESBL producing *E. coli* in chickens from 26/209 (12.7%) of small flocks sampled, which was less common compared to studies from other parts of the world (Doi et al., 2010; Overdevest et al., 2011). We

found that the CTX-M-1 β-lactamase was the predominant ESBL identified in small scale chickens in agreement with studies of agricultural animals from other countries (Belmar Campos et al., 2014; Girlich et al., 2007; Randall et al., 2011). This is in contrast to what is found in people in Canada where CTX-M-15 is most common (Peirano et al., 2010). One study in Canada identified a link between ceftiofur use in chicken population and increased resistance to ceftiofur in human population (Dutil et al., 2010), which indicates of having a similar resistance pattern in Canadian chickens and human isolates. However, our findings suggest that the ESBLs associated with chickens raised in small flocks are different than those found in the human population; this indicates that the zoonotic transmission of these genes occurs less commonly than would be expected based on phenotypic resistance alone. To demonstrate relatedness among isolates and find out resistant gene transfer pattern, we performed PFGE on these isolates. DNA fingerprinting demonstrated that these isolates were genetically diverse suggesting that resistance determinants in Ontario chickens might be transferred horizontally rather clonally, the CTX-M-1 gene is well recognized to be located on plasmids (Karim et al., 2001). These results were consistent with previous studies which found that CTX-M-1 producing *E. coli* isolates were genetically heterogenous (as determined by PFGE) and also concluded that plasmid mediated horizontal gene transfer plays an important role in the dissemination of the CTX-M-1 ESBL (de Been et al., 2014; Girlich et al., 2007; Liebana et al., 2006). Extra-intestinal pathogenic *E. coli* belonging to sequence type 131 (ST131) are a common cause of human urinary tract infections and are associated with CTX-M-15 production (Madigan et al., 2015). This strain has been previously from retail chicken meat in USA, Italy suggesting that chicken may be a potential reservoir of ST131 in the community, and therefore a zoonotic risk (Ghodousi et al., 2016;

Johnson et al., 2017). In the present study, none of the isolates tested by MLST were ST131 suggesting that this strain is uncommon among chickens from small flocks.

Our findings from small-scale flocks triggered an investigation to determine which broad-spectrum β-lactamases are found in *E. coli* from chickens raised in large commercial flocks. Furthermore, as the CIPARS program does not use any selective media, we sought to investigate this potential gap in the program's ability to detect important resistant determinants; selective media have previously been shown to be useful in the detection of ESBL producing *E. coli* in surveillance studies (European Centre for Disease Prevention and Control, 2014; Swarna et al., 2015). In our study, we found that the inclusion of selective media allowed us to detect a population of multidrug-resistant organisms which would have otherwise been missed. Despite the differences between small and large flocks, CTX-M-1 was also identified as predominant ESBL in this investigation. These findings suggest that despite differences between small and large-scale chicken flocks in Ontario, there may be a common source of ESBL genes possibly including: hatcheries, breeder farms or the environments.

Links between the use of antibiotics in chickens and the presence of resistant organisms have been previously described (Agersø et al., 2012; Cohen Stuart et al., 2012; Lowrance et al., 2007). Although we do not have sufficient antimicrobial usage data in chickens in Canada, one explanation for lower rates of resistance could be a low or decreasing rate of 3rd generation cephalosporin use in poultry (Chicken Farmers of Canada, 2017). As ceftiofur use has been decreased in Canada, thus it will be interesting to see rates of ceftiofur resistance and the prevalence of the ESBLs to demonstrate the link between AMU and AMR. Further, ceftiofur is categorized as Category I drug based on importance to human health (Government of Canada,

2009), continuous monitoring on antimicrobial resistance on this chicken population is extremely important to prevent and control any future outbreak.

Our studies had few limitations. We tested a selection of isolates by MLST, it might be possible that we were fail to detect *E. coli* ST131 from rest of the isolates where were not included. Further, isolates were selectively cultured on CHROMagar ESBL to phenotypically detect ESBL producers, thus this study was not able to provide actual AmpC producers rate in Canadian chickens.

4.2 Future directions

This study provides the first description of the presence of ESBL and AmpC type β-lactamases in *E. coli* isolated from chickens in Canada. Performing similar studies in other agricultural animals to determine the association of resistance pattern and resistance gene types between animal species will help to better understand the epidemiology of antimicrobial resistance. Future studies to identify risk factors associated with the dissemination of ESBLs would be beneficial to inform evidence-based policy to control the spread of these resistance determinants. Furthermore, determining the genetic relatedness of *E. coli* from small and large-scale chicken production may help to understand the epidemiology of resistance. Moreover, it will also be interesting to compare ESBL producing *E. coli* isolates from human and chicken population to find out any association in terms of resistance.

4.3 General conclusion

- The use of selective media in AMR studies can help to identify ESBL producing *E. coli* which could be missed.

- CTX-M-1 and CMY-2 were the mostly encountered ESBL and AmpC types in chickens from Canadian small and large-scale flocks.
- Horizontal gene transfer is playing an important role in transferring antimicrobial resistance determinants in Canadian chickens.
- Public health risk appears to be lower than previously assumed at least for ESBLs.

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